



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US99/23355 (22) International Filing Date: 07 October 1999 (07.10.1999) (30) Priority Data: 60/103,385 07 October 1998 (07.10.1998) US 60/105,805 27 October 1998 (27.10.1998) US 60/116,409 19 January 1999 (19.01.1999) US 60/136,260 27 May 1999 (27.05.1999) US 60/151,486 30 August 1999 (30.08.1999) US (60) Parent Application or Grant THE BOARD OF TRUSTEES OF THE UNIVERSITY OF ARKANSAS [/]; (). MANOLAGAS, Stavros, C. [/]; (). JILKA, Robert, L. [/]; (). WEINSTEIN, Robert, S. [/]; (). BELLIDO, Teresita [/]; (). BODENNER, Donald [/]; (). KOUSTENI, Stavroula [/]; (). KNOWLES, Sherry, M. ; ().		Published
(54) Title: METHOD AND COMPOSITIONS FOR INCREASING BONE MASS (54) Titre: PROCEDES ET COMPOSITIONS D'AUGMENTATION DE LA MASSE OSSEUSE (57) Abstract <p>The invention as disclosed provides a method to increase bone mass without compromising bone strength or quality, through the administration to a host of a compound that binds to the estrogen or androgen receptor without causing hormonal transcriptional activation.</p> (57) Abrégé <p>La présente invention concerne un procédé permettant d'augmenter la masse osseuse sans compromettre la qualité ou la résistance osseuse, en administrant à un hôte un composé se liant à un récepteur de l'oestrogène ou de l'androgène sans induire d'activation transcriptionnelle hormonale.</p>		

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METHOD AND COMPOSITIONS FOR INCREASING BONE MASS

BACKGROUND OF THE INVENTION

Federal Funding

This invention was funded in part through a grant from the National Institutes of Health. Therefore, the federal government has certain rights in this invention.

Field of the Invention

This invention is in the field of bone physiology, and in particular provides methods and compositions that include compounds to increase bone mass, *i.e.*, to achieve bone anabolism. The compounds bind to the estrogen or androgen receptor without causing significant hormonal transcriptional activation.

Description of the Related Art

Bones consist of living cells embedded within a matrix of proteins and minerals. Bones provide support and protection to the vital organs of the animal, and give strength and form to its structure.

Osteoporosis is a decrease in bone mass in combination with microarchitectural deterioration which leads to bone fragility and fractures. Treatments for osteoporosis have

5 historically focused on the prevention of further bone loss. In
contrast, a bone anabolic agent is one that substantially increases
bone mass. To date, while there have been several drugs
10 approved by the U.S. Food and Drug Administration for the
5 treatment of osteoporosis, it is believed that no drug has yet been
approved in the United States to be used as a bone anabolic agent,
15 for either humans or other animals. Bone is a dynamic tissue
which undergoes continual resorption and formation through a
remodeling process, which is accomplished by two types of cells:
20 10 osteoclasts, which erode cavities, and osteoblasts that synthesize
new bone matrix. Remodeling takes place mainly on the internal
surfaces of bone and it is carried out not by individual cells, but
25 rather by temporary anatomical structures, termed basic multi-
cellular units (BMUs), comprising teams of osteoclasts in the front
15 and osteoblasts in the rear. In an established BMU, bone
resorption and formation happens at the same time.

30 After osteoclasts stop resorbing bone, they die by
apoptosis and are quickly removed by phagocytes. During the
longer lifespan of the osteoblasts (about three months, as
35 compared to three weeks for osteoclasts), some osteoblasts
20 convert to lining cells that cover quiescent bone surfaces and some
are entombed within the mineralized matrix as osteocytes (Parfitt,
40 In: Bone, Telford and CRC Press, PP351-429, 1990). However, the
majority (65%) of osteoblasts that originally assembled at the
25 remodeling site die by apoptosis (Jilka et al, JBMR 13:793-802,
45 1998).

Most metabolic disorders of the adult skeleton result
from an imbalance between the resorption of old bone by

5 osteoclasts and its subsequent replacement by osteoblasts.
Changes in cell numbers, as opposed to individual cell activity
10 (Manolagas and Jilka, NEJM 332:305-311, 1995), appear to be the
cause of most metabolic bone diseases, including the three most
5 common forms of osteoporosis: osteoporosis due to sex steroid
deficiency in females and males (Jilka et al., Science 257:88-91,
15 1992; Jilka et al., JCI 101:1942-1950, 1998; Bellido et al., JCI
95:2886-2895, 1995; Weinstein et al., Endocrinology 138:4013-
4021, 1997); osteoporosis due to old age (Jilka et al., JCI 97:1732-
20 1740, 1996); and osteoporosis due to glucocorticoid-excess
(Weinstein et al., JCI 102:274-282, 1998; Weinstein et al, Bone,
23:S461, 1998; Bellido et al, Bone, 23:S324, 1998).

25 Agents that reduce bone turnover by inhibiting the
activation of bone remodeling (commonly but inaccurately
15 referred to as "antiresorptive") increase bone mass by a maximum
of 6-10%, and more typically, 2-3%, as measured by Dual Energy
30 X-Ray Absorptiometry (DEXA). Most of this increase is in the first
1-2 years and is due to contraction of the remodeling space.
Modest further increases may result from more complete
35 secondary mineralization. Improvement of focal balance due to
20 reduction of resorption depth has been demonstrated in animal
experiments, but not yet in human subjects. Regardless of the
40 mechanism, an increase of less than 10% will in almost all cases
fail to restore bone mass to its peak value and fail to reestablish
25 trabecular connectivity so that fracture risk will remain increased.

45 There are a wide variety of needs for bone anabolic
agents for humans as well as animals. Examples of uses for bone
anabolic agents in humans, besides patients with osteoporosis,
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5 include the strengthening of bone in healthy subjects who engage
in strenuous physical activities such as sports or manual labor,
and the strengthening of bone in persons who do not have
10 osteoporosis but might be subject to osteoporosis in the future
5 because the person is in a risk group for that disease. Other uses
for a bone anabolic agent in humans include the treatment of
15 persons who fail to obtain an adequate bone mass at the
completion of growth or persons who are born with unusually
fragile bones, persons who have a genetic predisposition to a bone
20 catabolic disease, or an orthopedic bone disease such as joint
degeneration, non-union fractures, orthopedic problems caused by
diabetes, periimplantitis, poor responses to bone grafts, implants,
25 fracture.

Likewise, there are many uses for bone anabolic
15 agents in animals. For example, it would be useful to increase the
bone mass in horses and dogs used for labor as well as those used
30 in sports such as racing. It would also be useful to increase the
bone mass in chickens and turkeys used in meat production to
maximize the amount of meat yield per animal.

20 There are currently ten classes of drugs that are used
in the treatment of osteoporosis: anabolic steroids,
bisphosphonates, calcitonins, estrogens/progestogens, Selective
40 Estrogen Receptor Modulators (SERMs) such as raloxifene,
phytoestrogen, parathyroid hormone ("PTH"), fluoride, Vitamin D
25 metabolites, and calcium preparations. No compound within these
45 classes has been approved as a bone anabolic agent.

Anabolic Steroids (Androgens)

5 Anabolic steroids (androgens) have been known to
build muscle mass in the host. However, there has been no
reported evidence that they function as bone anabolic agents as
10 defined herein (Snyder et al, JCEM 84:1966-1972, 1999).

5 Androgens are typically used as a replacement therapy for male
hypogonadal disorders and they are used in adolescent males with
15 a history of delayed puberty or growth. Androgens can produce
significant side effects when taken over a period of time, including
water retention, jaundice, decreased high density lipoprotein and
20 increased low density lipoprotein, hepatic toxicity (most usually
associated with the 17 α -alkylated androgens), hepatic carcinoma,
increased risk of cardiovascular disease, and when taken in large
25 dosages, irrationality, psychotic episodes, violent behavior, and
death. U.S. Patent No. 5,565,444 discloses the use of an androgen
15 for the treatment of bone loss or for increasing bone mass.

30 Calcitonin

Endogenous calcitonin is a polypeptide hormone
involved in the regulation of calcium and bone metabolism. Forms
used therapeutically include calcitonin (pork), extracted from pig
35 thyroid, a synthetic human calcitonin; elcatonin, a synthetic
analogue of eel calcitonin; and salcatonin, a synthetic salmon
calcitonin. They all have the property of lowering plasma-calcium
40 concentration by diminishing the rate of bone resorption.
Calcitonins are typically administered subcutaneously or by
25 intramuscular injection.

45 Bisphosphonates

Bisphosphonates have been widely used to treat
osteoporosis. The bisphosphonate disodium etidronate has similar
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5 effects on bone mass and fractures in established osteoporosis to
those of calcitonin, but cannot be given for a prolonged period
because of the risk of osteomalacia. Bisphosphonate alendronate
10 treatment at a dose of 10 mg/day results in a 5% increase in
5 spinal bone mineral density (BMD) over the first year (Dempster,
Exploiting and Bypassing the Bone Remodeling Cycle to Optimize
the Treatment of Osteoporosis, Journal of Bone and Mineral
15 Research, Volume 12, Number 8, 1997, pages 1152-1154). BMD
continues to increase, albeit at a slower rate, at this site during the
20 second and third years of treatment. The magnitude and duration
of the increase in BMD has led to speculation that alendronate is
doing more than simply reducing remodeling space and that it
25 may possess anabolic activity. The bisphosphonate etidronate
reduced resorption depth in human iliac trabecular bone by
15 almost 30% after one year of treatment, but no such data are yet
available for alendronate. Etidronate did not change the thickness
30 of trabecular packets, but recent studies in osteoporotic women
suggest that this is increased after two years of alendronate
treatment at 10 and 20 mg/day. This result was not confirmed
35 after three years of treatment.
20

In another article, Dempster (Dempster D.W., New
concepts in bone remodeling, *In: Dynamics of Bone and Cartilage*
40 *Metabolism*, Chapter 18, pp.261-273, Acad. Press, 1999) confirms
that the potential for an agent that can increase bone mass and
25 hence reverse the skeletal defect in patients with osteoporosis is
great, particularly if in doing so it also repairs microarchitectural
45 damage. He notes that estrogens and calcitonin primarily stabilize
bone mass and prevent further loss of bone, although a transient
50

5 small increment in mass is often reported, particularly in patients
with elevated levels of bone remodeling. Dempster et al conclude
that this is not a true anabolic effect but is related to the temporal
10 effects on turnover in which resorption declines initially followed
5 by a reduction in formation that may take several months.

Albeit, bisphosphonates have anti-apoptotic effects on
15 osteoblasts and osteocytes (Plotkin et al. Bone, 23:S157, 1998).
Significantly, the anti-apoptotic effect of bisphosphonates *in vitro*
is achieved with doses 100-1000 lower than the doses at which
20 these same agents inhibit osteoclast activity; and additionally can
be demonstrated with bisphosphonates that do not block
osteoclast activity at all (compound IG9204). U.S. Patent No.
4,870,063 discloses a bisphosphonic acid derivative to increase
25 bone mass. U.S. Patent Nos. 5,532,226 and 5,300,687 describe the
15 use of trifluoromethylbenzylphosphonates to increase bone mass.
U.S. Patent No. 5,885,973 to Papapoulos, et al, discloses a bone
mass anabolic composition that includes olpandronate, which is a
30 bisphosphonate.

35 Estrogens/progestogens

20 Estrogens/progestogens (anti-remodeling and anti-
resorptive compounds) as a class have not to date been shown to
increase bone mass by more than 10%, but instead have been
40 used to retard the effect of osteoporosis. Estrogens are currently
the most effective method of preventing osteoporosis in
25 postmenopausal women.

45 U.S. Patent No. 5,183,815 discloses the use of a
steroidal hormone covalently linked to a hydroxy alkyl-1,1-
bisphosphonate. U.S. Patent No. 5,843,934 claims that an estrogen
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5 having insubstantial sex-related activity can be administered to a
patient to retard the adverse effects of osteoporosis in a male or
female. The '934 patent does not address how to select a
10 compound to increase bone mass, but instead teaches how to
5 retard the effect of bone loss. WO 98/22113 filed by the
University of Florida Research Foundation, Inc. discloses methods
to utilize an isomer of an estrogen compound to confer
15 cytoprotection on a population of cells associated with an ischemic
event.

10 Phytoestrogens

Little is known about the actions of phytoestrogens on
bone (Fitzpatrick, L.A., Mayo Clinic Proceedings, 74:601-607,
1999). Soy protein did not prevent increased bone turnover in
25 cynomolgus monkeys; they actually increased it. However, BMD
15 declined after two years in postmenopausal women taking only
calcium but did not change in those receiving ipriflavone.
Isoflavone significantly increased spinal BMD in postmenopausal
women after 6 months of 40 mg/day of soy protein
supplementation (containing 90 mg isoflavones) but not with
35 lower doses (56 mg/day) (Feinkel, E. Lancet, 352:762, 1998).

20 Parathyroid Hormone (PTH)

Daily injections of parathyroid hormone (PTH), a
40 agent known for its role in calcium homeostasis, increases bone
mass in animals and humans, as does the related PTH-related
25 hormone PTHrP, the only other known ligand of the PTH receptor.
45 Whereas increased prevalence of apoptosis of osteoblasts and
osteocytes are key pathogenic mechanisms for osteoporosis
(Weinstein et al., J Clin Invest, 102:274-282, 1998; Weinstein et al,

5 Bone, 23:S461, 1998; Bellido et al, Bone, 23:S324, 1998), the
reverse, *i.e.*, postponement of osteoblast apoptosis, is the principal,
if not the sole, mechanism for the anabolic effects of intermittent
10 parathyroid hormone administration on bone (Jilka et al., J. Clin.
5 Invest. 104:439-446, 1999). The increased bone mineral density,
osteoblast perimeter and bone formation rate that occur with
intermittent PTH administration in mice happen without a change
15 in osteoblast production. Instead, the anabolic effect of the drug is
due to decreased prevalence of osteoblast apoptosis from 1.7-2.2%
20 to as little as 0.1-0.4%, while the osteocytes in the newly made
lamellar cancellous bone are closer together and more numerous
than those found in the animals receiving vehicle alone. The
25 closely spaced, more numerous osteocytes are the predictable
consequence of protecting osteoblasts from apoptosis. The anti-
15 apoptotic effect of PTH on osteoblasts as well as osteocytes has
been confirmed *in vitro* using primary bone cell cultures and
30 established cell lines.

The use of teriparatide (the 1-34 amino acid fragment
of human parathyroid growth hormone) to stimulate bone
35 formation has also been investigated; teriparatide administered as
20 daily injections has been reported to selectively increase the
trabecular bone density of the spine in osteoporotic patients.

40 U.S. Patent No. 5,510,370 discloses the use of a
combination of PTH and raloxifene to increase bone mass. U.S.
25 Patent No. 4,833,125 discloses the use of PTH in combination with
45 either a hydroxylated vitamin D derivative, or a dietary calcium
supplement.

Calcium Preparations

Calcium preparations, while useful as a dietary supplement for persons who are calcium deficient, have not been shown effective to increase bone mass. However, they may reduce the rate of bone loss. U.S. Patent No. 5,618,549 (a calcium salt) describes the use of calcium.

Fluoride

The most thoroughly studied anabolic agent, sodium fluoride, can increase vertebral bone mass by 10% a year for at least four years but there is controversy about the quality of the bone formed. Sodium fluoride has not been approved as a bone anabolic agent. It has been difficult to establish anti-fracture efficacy because of serious qualitative abnormalities. First, much of the new bone is initially woven rather than lamellar. Second and more important, there is severe impairment of bone mineralization, in spite of sodium fluoride's effectiveness in increasing bone mass.

U.S. Patent No. 5,071,655 discloses a composition to increase bone mass that includes a fluoride source and a mitogenic hydantoin.

SERMs

SERMs such as tamoxifen and raloxifene have also been used to treat osteoporosis. A recent study carried out with raloxifene indicated that after three years of treatment, women on raloxifene had 30-50% fewer spinal fractures, and had 2-3% increase in bone density in their hips and spine, but showed no fewer nonspinal fractures, a category that includes hip fractures (Ettinger, B., JAMA, 282:637-645, 1999).

5 U.S. Patent No. 4,970,237 discloses the use of
clomiphene to increase bone mass in premenopausal women.

Vitamin D derivatives

10 There have been conflicting reports about the value of
5 Vitamin D or its derivatives on bone loss and bone anabolism.
Some studies on the hormonal metabolite of vitamin D, calcitriol,
15 have reported an increase in spinal bone density, but others have
found no effect.

The following patents describe the use of Vitamin D
10 derivatives to treat bone disease: U.S. Patent Nos. 4,973,584;
20 5,750,746; 5,593,833; 5,532,391; 5,414,098; 5,403,831; 5,260,290;
5,104,864; 5,001,118; 4,973,584; 4,619,920; and 4,588,716.

Other Compounds

25 The following patents disclose the use of other
15 compounds for the treatment of bone disease: U.S. Patent Nos.
5,753,649 and 5,593,988 (azepine derivative); 5,674,844
30 (morphogen); 5,663,195 (cyclooxygenase-2 inhibitor); 5,604,259
(ibuprofen or flurbiprofen); 5,354,773 (bafilomycine); 5,208,219
(activin); 5,164,368 (growth hormone releasing factor); and
35 20 5,118,667, 4,870,054 and 4,710,382 (administration of a bone
growth factor and an inhibitor of bone resorption).

40 U.S. Patent No. 5,859,001 discloses the use of non-
estrogen compounds having a terminal phenol group in a four-ring
cyclopentanophenanthrene compound structure to confer
25 neuroprotection to cells.

45 U.S. Patent No. 5,824,672 discloses a method for
preserving tissues during transplantation procedures that includes

5 administering an effective dose of a cyclopentanophenanthrene
compound having a terminal phenol A ring.

10 WO 98/31381 filed by the University of Florida
Research Foundation, Inc. discloses a method for enhancing the
5 cytoprotective effect of polycyclic phenolic compounds on a
population of cells that involves the steps of administering a
combination of polycyclic phenolic compounds and anti-oxidants
15 to achieve an enhanced effect. One disclosed combination is
glutathione and estrogen.

20 10 It is an object of the present invention to provide a
method to increase bone mass in a host by at least 10% per year
without a loss in bone strength (defined by fracture incidence *in*
25 *vivo* and mechanical strength *in vitro*) and/or deterioration of
bone quality (as defined by abnormal collagen orientation and
15 excessive accumulation of unmineralized bone matrix, determined,
for example, with histomorphometry).

30 It is another object of the present invention to provide
a method to rebuild strong bones instead of preventing further
loss of bone.

35 20 It is a further object of the present invention to
provide a method to select compounds that increase bone mass in
a host at least 10% per year without a loss in bone strength or
40 quality.

45 25 It is a still further object of the present invention to
provide a method to increase bone strength by at least 20%.

SUMMARY OF THE INVENTION

In a first embodiment, a method for increasing bone mass in a host at least 10% without a loss in bone strength or quality is provided that includes administering an effective amount of a compound that (i) binds to the estrogen α or β receptor (or the equivalent receptor in the host animal) with an association constant of at least 10^8 M^{-1} , and preferably, at least 10^{10} M^{-1} ; (ii) (a) induces estrogenic gene transcriptional activity at a level that is no greater than 10% that of 17 β -estradiol, and preferably no greater than 5, 1 or even 0.1% that of 17 β -estradiol when administered *in vivo* at a dosage of at least 0.1 ng/kg body weight or *in vitro* in osteoblastic or osteocytic cells with natural estrogen receptors or cells transfected with estrogen receptors or (b) induces an increase in uterine weight of no more than 10% that of 17 β -estradiol (or the equivalent compound in a host animal); (iii) induces the phosphorylation of extracellular signal regulated kinase (ERK) when administered *in vivo* at a dosage of at least 0.1 ng/kg body weight or *in vitro* in osteoblastic cells with natural estrogen receptors or cells transfected with estrogen receptors; and (iv) has an anti-apoptotic effect on osteoblasts and osteocytes at an *in vivo* dosage of at least 0.1 ng/kg body weight or *in vitro* in osteoblastic or osteocytic cells with natural estrogen receptors or cells transfected with the estrogen receptor. In another aspect of this first embodiment of this invention, the compound is not an estrogen compound, as that term is defined below. In yet another aspect of this first embodiment, the compound is an estrogen compound which is converted to a nonestrogen by attaching a

5 substituent which prevents the compound from entering the cell
but does not significantly affect the binding of the compound to
the estrogen cell-surface receptor.

10 In a second embodiment, a method for increasing bone
5 mass in a host at least 10% without a loss in bone strength or
quality is provided that includes administering an effective
amount of a compound that (i) binds to the androgen receptor (or
15 the equivalent receptor in the host animal) with an association
constant of at least 10^8 M^{-1} , and preferably, at least 10^{10} M^{-1} ; (ii)
20 (a) induces androgenic gene transcriptional activity at a level that
is no greater than 10% that of testosterone, and preferably no
greater than 5, 1 or even 0.1% that of testosterone when
25 administered *in vivo* at a dosage of at least 0.1 ng/kg body weight
or *in vitro* in osteoblastic cells with the natural androgen receptor
15 or cells transfected with the androgen receptor or (b) induces an
increase in muscle weight of no more than 10% that which is
induced by testosterone (or the equivalent compound in a host
30 animal); (iii) induces the phosphorylation of extracellular signal
regulated kinase (ERK) when administered *in vivo* at a dosage of
35 at least 0.1 ng/kg body weight or *in vitro* in osteoblastic cells with
the natural androgen receptor or cells transfected with the
androgen receptor; and (iv) has an anti-apoptotic effect on
40 osteoblasts and osteocytes at an *in vivo* dosage of at least 0.1
ng/kg body weight or *in vitro* in osteoblastic cells with the natural
25 androgen receptor or transfected with the androgen receptor. In
another aspect of the second embodiment, the compound is not an
45 androgen. In yet another aspect of this second embodiment, the
compound is an androgen compound which is converted to a
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5 nonandrogen by attaching a substituent which prevents the
compound from entering the cell but which does not significantly
affect the ability of the compound to bind to the androgen cell-
10 surface receptor.

5 In other aspects of the first or second embodiment of
this invention, the compound has a pro-apoptotic effect on
osteoclasts at an *in vivo* dosage of at least 0.1 ng/kg body weight,
15 or in osteoclastic cells with natural estrogen receptors or cells
transfected with estrogen receptors.

10 The disclosed invention is based on the fundamental
discovery that bone loss occurs because of an increase in
osteoblast and perhaps osteocyte apoptosis, which can be inhibited
25 by a compound that binds to an estrogen or androgen receptor,
which induces the phosphorylation of ERKs without significant
15 hormonal transcriptional activation. The discovery of this
fundamental pathway allows the selection of compounds which
30 provide a maximum effect on bone mass and strength.

Therefore, in a third embodiment, a method for
selecting a compound that increases bone mass in a host at least
35 20 10% without a loss in bone strength or quality is provided that
includes evaluating whether the compound (i) binds to the
estrogen or androgen receptor (or the equivalent receptor in the
40 host animal) with an association constant of at least 10^8 M^{-1} , and
preferably, at least 10^{10} M^{-1} ; (ii) (a) induces estrogenic or
25 androgenic gene transcriptional activity at a level that is no
45 greater than 10% that of 17β -estradiol or testosterone, and
preferably no greater than 5, 1 or even 0.1% that of 17β -estradiol
or testosterone, as appropriate, when administered *in vivo* at a
50

5 dosage of at least 0.1 ng/kg body weight or *in vitro* in osteoblastic
cells with the natural androgen or estrogen receptor or cells
transfected with the androgen or estrogen receptor or (b) induces
10 an increase in uterine weight of no more than 10% that which is
5 induced by 17 β -estradiol or muscle weight of no more than 10%
that which is induced by testosterone (or the equivalent
15 compound in a host animal); (iii) induces the phosphorylation of
extracellular signal regulated kinase (ERK) when administered *in*
vivo at a dosage of at least 0.1 ng/kg body weight or *in vitro* in
20 10 osteoblastic or osteocytic cells with the natural androgen or
estrogen receptor or cells transfected with the androgen or
estrogen receptor; and (iv) has an anti-apoptotic effect on
25 osteoblasts and osteocytes at an *in vivo* dosage of at least 0.1
ng/kg body weight or *in vitro* in osteoblastic and osteocytic cells
15 with the natural androgen or estrogen receptor or cells transfected
with the androgen or estrogen receptor.

30 Estrogenic compounds like 17 α -estradiol and synthetic
polycyclic phenols, such as estratriene-3-ol inhibit osteoblast and
osteocyte apoptosis *in vitro*. Yet unlike the classical mechanism of
35 20 estrogen receptor action that involves direct or indirect interaction
with the transcriptional apparatus, the receptor-dependent anti-
apoptotic effects of these compounds are nongenomic, as they are
40 due to rapid (within 5 minutes) phosphorylation of ERKs.
Estratriene-3-ol increases bone mass in both estrogen-replete and
25 45 estrogen-deficient mice. Esstratriene-3-ol, when given in low
doses, has little effect on estrogenic-type activity but also has
little effect on bone mass. As the dosage increases, both effects
increase. To optimize the use of this compound or others

5 exhibiting this type of activity, one can derivatize the compound
to preserve the estrogen-binding activity and decrease the
transcriptional activity as described in detail herein, including by
10 attaching a substituent or moiety that inhibits cell penetration.

5 Compounds selected according to the criteria provided
herein can also be used for the augmentation of bone mass and/or
fracture prevention in diseases characterized by low bone mass
15 and increased fragility. The compounds can also be used to treat
bone disease states in which osteoblastogenesis is decreased, such
20 as senile osteoporosis, and glucocorticoid-induced osteoporosis--
especially in growing children and adolescents, during which time
in whom interfering with bone remodeling is detrimental.

15 BRIEF DESCRIPTION OF THE DRAWINGS

30 The Figures provided herein illustrate embodiments of
the invention and are not intended to limit the scope of the
invention.

35 20 Figure 1 provides nonlimiting examples of one class
of compounds that can be used to increase bone mass without
adversely affecting bone strength.

40 Figure 2 is a bar chart graph of the degree of
apoptosis of osteoblasts and osteocytes in murine vertebral bone
25 as a function of estrogen deficiency. Swiss Webster mice (four
45 months old) were ovariectomized. Twenty eight days later, the
animals were sacrificed, vertebrae were isolated, fixed and
embedded, and then undecalcified in methacrylate. The

prevalence of osteoblast and osteocyte apoptosis was determined by the TUNEL method with CuSO_4 enhancement, and was found to be dramatically increased following loss of estrogen. *** $P < 0.00001$; * $P < 0.0382$.

Figure 3 is a series of bar chart graphs which illustrate the percentage of Etoposide-induced osteoblast apoptosis versus the log of the concentration of added estrogens 17β -estradiol, 17β -estradiol-BSA, 17α -estradiol, and estratriene-3-ol. Osteoblastic cells derived from murine calvaria were pretreated with the sterols for 1 hour before the addition of the pro-apoptotic agent, etoposide. Apoptosis was determined after 6 hours by trypan blue uptake (Jilka et al, J.Bone and Min. Res. 13:793:802, 1998). * indicates $p < 0.05$ versus etoposide alone, by analysis of variance (ANOVA) (Student-Newman-Keuls method).

Figure 4 is a series of bar chart graphs of the inhibition of etoposide-induced apoptosis of osteocytes (MLO-Y4) by 17β -estradiol, 17β -estradiol-BSA, 17α -estradiol, and estratriene-3-ol. Cells were pretreated with the indicated concentrations of the compounds for 1 hour before the addition of the pro-apoptotic agent etoposide. Apoptosis was determined after 6 hour by trypan blue uptake as described in Figure 3. * indicates $p < 0.05$ versus etoposide alone, by ANOVA (Student-Newman-Keuls method).

Figure 5 is a series of bar chart graphs that indicates that the anti-apoptotic effect of 17β -estradiol, 17β -estradiol-BSA, 17α -estradiol, and estratriene-3-ol (E-3-ol) on etoposide-induced apoptosis of osteoblasts is abrogated by the estrogen receptor antagonist, ICI182,780. Osteoblastic cells derived from murine

5 calvaria were pretreated for 1 hour with the pure receptor
antagonist ICI182,780 (10^{-7} M) before the addition of the test
agents (10^{-8} M). Apoptosis was induced and quantified as
10 described in Figure 3. * indicates $p < 0.05$ versus etoposide alone, by
5 ANOVA (Student-Newman-Keuls method).

Figure 6 is a series of bar chart graphs that indicates
15 that the anti-apoptotic effect of 17β -estradiol, 17β -estradiol-BSA,
 17α -estradiol, and estratriene-3-ol (E-3-ol) on MLO-Y4 osteocytic
cells is abrogated by the estrogen receptor antagonist, ICI182,780.
20 MLO-Y4 cells were pretreated for 1 hour with the pure receptor
antagonist ICI182,780 (10^{-7} M) before the addition of the test
agents (10^{-8} M). Apoptosis was induced and quantified as
25 described in Figure 3. * indicates $p < 0.05$ versus etoposide alone, by
ANOVA (Student-Newman-Keuls method).

15 Figure 7 is a series of bar chart graphs which
demonstrate that estrogen receptor α or β is required for the anti-
30 apoptotic effects of 17β -estradiol, 17α -estradiol, and estratriene-
3-ol on the etoposide-induced apoptosis of osteoblasts. CMV
promoter alone and CMV promoter-driven cDNA for mER α or mER β
35 were stably transfected into HeLa cells. Subconfluent cultures
20 were treated for 1 hr with 10^{-8} M 17α -estradiol, 17β -estradiol, or
estratriene-3-ol followed by a 6 hr incubation with etoposide
40 (5×10^{-5} M). Cells were trypsinized, pelleted and trypan blue
positive cells enumerated. Each bar represents mean of duplicate
25 experiments \pm SEM. *P < 0.02 versus etoposide alone.

Figure 8 is Western blot which demonstrates that
17 β -estradiol, 17α -estradiol, 17β -estradiol-BSA or estratriene-3-ol
50 activate the extracellular signal regulated kinases (ERKs). MLO-Y4

5 osteocytic cells were incubated for 25 minutes in serum-free
medium. Subsequently, 17β -estradiol, 17α -estradiol, 17β -
10 estradiol-BSA or estratriene-3-ol (10^{-8} M) were added and cells
5 prepared and proteins were separated by electrophoresis in
polyacrylamide gels and transferred to PVDF membranes.
15 Western blotting was performed using a specific antibody
recognizing phosphorylated ERKs 1 and 2, followed by reblotting
with an antibody recognizing total ERKs. Blots were developed by
20 enhanced chemiluminescence.

Figure 9 is a Western blot which demonstrates that
the effect of estrogenic compounds on the activation of ERK1/2 is
25 blocked by the specific inhibitor of ERK kinase, PD98059. MLO-Y4
cells were incubated for 25 minutes in serum-free medium in the
15 presence or absence of 50 μ M PD98059. Subsequently, 17β -
estradiol, 17α -estradiol, 17β -estradiol-BSA or estratriene-3-ol (10^{-8}
30 M) were added and cells incubated for an additional 5 min. Cell
lysates were prepared and proteins were separated by
electrophoresis in polyacrylamide gels and transferred to PVDF
35 membranes. Western blotting was performed using a specific
20 antibody recognizing phosphorylated ERKs 1 and 2, followed by
reblotting with an antibody recognizing total ERKs. Blots were
40 developed by enhanced chemiluminescence.

Figure 10 is a series of bar chart graphs which
25 demonstrate that the specific inhibitor of ERK activation, PD98059,
45 abolishes the anti-apoptotic effect of 17β -estradiol and related
compounds. MLO-Y4 osteocytic cells were pretreated for 1 hour
with 50 μ M PD98059 before the addition of 10^{-8} M 17β -estradiol,
50

5 17 α -estradiol, or 17 β -estradiol-BSA. Apoptosis was induced by
incubation with the pro-apoptotic agent dexamethasone for 6 hour
and quantified as described in Figure 3. * indicates $p < 0.05$ versus
10 the corresponding control group without dexamethasone, by
5 ANOVA (Student-Newman-Keuls method).

Figure 11 illustrates that unlike 17 β estradiol,
15 estratriene-3-ol does not transactivate an estrogen response
element through ER α . The human ER α was overexpressed in 293
cells lacking ER α along with a reporter construct containing 3
20 10 copies of an estrogen response element driving the luciferase
gene. Light units were counted and normalized to coexpressed b-
galactosidase activity to control for differences in transfection
25 efficiency. Results represent percent stimulation compared to ER α
transfected cells, but not treated with the two agents. Each bar
15 represents mean of duplicate experiments +/- SEM. * $p < 0.001$ vs.
30 cells not exposed to the sterols.

Figure 12 is an illustration of the chemical structures
of certain 3-ring compounds: [2S-(2a,4a α ,10a β)]-
35 1,2,3,4,4a,9,10,10a-octahydro-7-hydroxy-2-methyl-2-
20 phenanthrenemethanol (PAM) and [2S-(2a,4a α ,10a β)]-
1,2,3,4,4a,9,10,10a-octahydro-7-hydroxy-2-methyl-2-
40 phenanthrenecarboxaldehyde (PACA).

Figure 13 illustrates the generalized core ring
structures with numbered carbons (Figure 13a) 4-ring structure,
45 25 (Figure 13b) 3-ring structure, (Figure 13c) 2-ring structure
(fused), and (Figure 13d) 2-ring structure (non-fused).

Figure 14 is an illustration of three mechanisms of
50 estrogen activity: Figure 14A (anti-apoptotic effect of estrogen),

5 **Figure 14B** (anti-remodeling effect of estrogen) and **Figure 14C**
(feminizing effect of estrogen).

10 **Figure 15** compares the activity of the anti-
resorptive (*e.g.*, 17 β -estradiol) versus non-anti-resorptive agents
5 [*e.g.*, estratriene-3-ol or intermittent PTH] on osteoblast and
osteocyte apoptosis. Bone formation occurs only on sites of
previous osteoclastic bone resorption, *i.e.*, on sites undergoing
15 remodeling. Each remodeling cycle is a transaction that, once
consummated, is irrevocable. As shown in the right panel, agents
20 with anti-apoptotic properties that do not have anti-
resorptive/anti-remodeling properties rebuild more bone and
therefore, increase the overall bone mass because they will not
25 decrease the number of the remodeling units (*i.e.*, the number of
transactions). In addition, by decreasing the prevalence of
15 osteoblast apoptosis, the active compounds expand the pool of
mature osteoblasts at sites of new bone formation and allow these
30 cells more time to make bone. Moreover, by upholding the
osteocyte-canalicular network by preventing osteocyte apoptosis,
35 both classical antiresorptive agents like 17 β -estradiol and agents
20 that are not anti-resorptives are expected to have anti-fracture
efficacy over and above that resulting from their effects on bone
mass.

40 **Figure 16A** is a table of examples of R1 and R2
substitutions on the compound illustrated in Figure 1.

45 **Figure 16B** provides the molecular structures of α
and β estradiol.

50 **Figure 17** provides the chemical structures of
estratrienes with anti-apoptotic properties.

5 **Figure 18** provides the chemical structures of estradiol, phenol and diphenols with anti-apoptotic properties.

10 **Figure 19** depicts the effect of 17β estradiol on the transcriptional activity of a minimal ERE containing gene promoter
5 and the blockade of this effect by a peptide (α II) recognizing the ligand-induced specific conformational change of the estrogen receptor protein. 293, human kidney cells, were transiently
15 transfected with a plasmid carrying the ER-specific α II peptide with the GAL4-DNA binding domain inserted upstream of the peptide sequence, an ERE/IL-6 promoter-driven luciferase
20 10 reporter plasmid and a β -galactosidase (β -gal)-containing plasmid. The ERE-luciferase construct carried three copies of the *Xenopus vitellogenin* ERE driving the luciferase gene in the pGL3-Basic
25 vector (Promega). * indicates $p < 0.05$ versus cells transfected with the peptide α II, by ANOVA (Student-Newman-Keuls method).
15

30 **Figure 20** depicts the effect of 17β estradiol on the transcriptional activity of the IL-6 promoter and the blockade of this effect by a peptide (α II) recognizing the ligand-induced
35 specific conformational change of the estrogen receptor protein. 20 The IL-6-luciferase plasmid carried 225bp of the proximal IL-6 promoter cloned upstream of the luciferase gene in pGL3-Basic.
40 The α II peptide inhibited the transcriptional effects of estrogen on the ERE-dependent transcription model. α II was also shown to block transcription when mediated via protein/protein interaction
45 25 between the ER and another transcription factor on the IL-6 gene model. * indicates $p < 0.05$ versus cells transfected with the peptide α II, by ANOVA (Student-Newman-Keuls method).
50

5 **Figure 21** demonstrates the anti-apoptotic effect of
17 β estradiol conjugated with BSA and the lack of inhibition of
this particular effect by the conformation sensitive peptide α II.
10 The effect of the peptide on apoptosis was assayed using
5 etoposide as the apoptotic stimulus. Upon etoposide treatment,
cells that had been transfected with the ER and treated with 17 β -
15 BSA were protected from apoptosis. Following co-transfection of
the GAL4-driven peptide, cells remained resistant to etoposide-
induced apoptosis indicating that the peptide did not inhibit the
20 10 protective, anti-apoptotic action of the ER (Figure 21). * indicates
p<0.05 versus cells transfected with the peptide α II, by ANOVA
(Student-Newman-Keuls method).

15 **DETAILED DESCRIPTION OF THE INVENTION**

30 The invention as disclosed provides a method to
increase bone mass without compromising bone quality, through
the administration to a host of an effective amount of a compound
35 20 that binds to the estrogen or androgen receptor so as to trigger the
anti-apoptotic signalling pathway, but with minimal or no
40 resultant transcriptional activity.

45 25 In an optimal embodiment using this invention, an
anabolic effect will be established by demonstrating increased
bone formation, assessed by double tetracycline labeling
(Weinstein R.S. *In Disorders of Bone and Mineral Metabolism* (eds.
50 Coe and Favus) Raven Press, 1992, pp. 455-474) and a continuous
increase in BMD, assessed by DEXA (Jilka et al. *J. Clin. Invest.*

5 97:1732-1740, 1996) for at least five years, along with increased,
or at least no decreased quality or strength.

10 This invention is based on the fundamental discovery
that bone loss occurs because of an increase in osteoblast
5 apoptosis, which can be inhibited by a compound that binds to an
estrogen or androgen receptor (which induces the phosphorylation
of ERKs) with minimal or no resultant transcriptional activity. The
15 discovery of this fundamental pathway allows the selection of
compounds which provide a maximum effect on bone mass and
10 strength.

Therefore, in a first embodiment, a method for
increasing bone mass in a host at least 10% without a loss in bone
quality or strength is provided that includes administering an
25 effective amount of a compound that (i) binds to the estrogen α or
15 β receptor (or the equivalent receptor in the host animal) with an
association constant of at least 10^8 M^{-1} , and preferably, at least
30 10^{10} M^{-1} ; (ii) (a) induces estrogenic gene transcriptional activity at
a level that is no greater than 10% that of 17 β -estradiol, and
preferably no greater than 5, 1 or even 0.1% that of 17 β -estradiol
35 when administered *in vivo* at a dosage of at least 0.1 ng/kg body
weight or *in vitro* in cells with natural estrogen receptors or
transfected with estrogen receptors or (b) induces an increase in
40 uterine weight of no more than 10% that of estrogen (or the
equivalent compound in a host animal); (iii) induces the
25 phosphorylation of extracellular signal regulated kinase (ERK)
when administered *in vivo* at a dosage of at least 0.1 ng/kg body
weight or at a concentration of 10^{-11} to 10^{-7} M *in vitro* in cells with
45 natural estrogen receptors or transfected with estrogen receptors.;

5 and (iv) has an anti-apoptotic effect on osteoblasts and osteocytes
at an *in vivo* dosage of at least 0.1 ng/kg body weight or at a
concentration of 10^{-11} to 10^{-7} M *in vitro* in cells with natural
10 estrogen receptors or transfected with estrogen receptors. In
5 another aspect of this first embodiment of this invention, the
compound is not an estrogen compound, as that term is defined
herein. In another aspect of this first embodiment, the compound
15 is an estrogen compound which is converted to a nonestrogen by
attaching a substituent which prevents the compound from
20 10 entering the cell, but which does not significantly affect the
binding of the compound to the estrogen cell-surface estrogen
receptor.

25 In a second embodiment, a method for increasing bone
mass in a host at least 10% per year without a loss in bone
15 strength or quality is provided that includes administering an
effective amount of a compound that (i) binds to the androgen
30 receptor (or the equivalent receptor in the host animal) with an
association constant of at least 10^8 M⁻¹, and preferably, at least
 10^{10} M⁻¹; (ii) (a) induces androgenic gene transcriptional activity at
35 20 a level that is no greater than 10% that of testosterone, and
preferably no greater than 5, 1 or even 0.1% that of testosterone
when administered *in vivo* at a dosage of at least 0.1 ng/kg body
40 weight or *in vitro* in cells with the natural androgen receptor or
transfected with the androgen receptor or (b) induces an increase
25 in muscle weight of no more than 10% that which is induced by
45 testosterone (or the equivalent compound in a host animal); (iii)
induces the phosphorylation of extracellular signal regulated
kinase (ERK) when administered *in vivo* at a dosage of at least 0.1
50

5 ng/kg body weight, or at a concentration of 10^{-11} to 10^{-7} M *in vitro*
in cells with the natural androgen receptor or transfected with the
androgen receptor; and (iv) has an anti-apoptotic effect on
10 osteoblasts and osteocytes at an *in vivo* dosage of at least 0.1
5 ng/kg body weight or at a concentration of 10^{-11} to 10^{-7} M *in vitro*
in cells with the natural androgen receptor or transfected with the
15 androgen receptor. In another aspect of the second embodiment,
the compound is not an androgen. In another aspect of this
second embodiment, the compound is an androgen compound
20 10 which is converted to a nonandrogen by attaching a substituent
which prevents the compound from entering the cell containing
the cell-surface androgen receptor.

25 In other aspects of the first or second embodiment of
this invention, the compound also has a pro-apoptotic effect on
15 osteoclasts at an *in vivo* dosage of at least 0.1 ng/kg body weight
or *in vitro* in cells with the natural androgen receptor or
30 transfected with the androgen receptor.

Therefore, in a third embodiment, a method for
selecting a compound that increases bone mass in a host at least
35 20 10% without a loss in bone strength or quality is provided that
includes evaluating whether the compound (i) binds to the
estrogen or androgen receptor (or the equivalent receptor in the
40 host animal) with an association constant of at least 10^8 M⁻¹, and
preferably, at least 10^{10} M⁻¹; (ii) (a) induces estrogenic or
25 androgenic gene transcriptional activity at a level that is no
45 greater than 10% that of testosterone or 17 β -estradiol, and
preferably no greater than 5, 1 or even 0.1% that of 17 β -estradiol
or testosterone, as appropriate, when administered *in vivo* at a
50

dosage of at least 0.1 ng/kg body weight or at a concentration of 10^{-11} to 10^{-7} M or *in vitro* in cells with the natural androgen or estrogen receptor or transfected with the androgen or estrogen receptor or (b) induces an increase in uterine or muscle weight, as appropriate, of no more than 10% that which is induced by 17 β -estradiol or testosterone (or the equivalent compound in a host animal); (iii) induces the phosphorylation of extracellular signal regulated kinase (ERK) when administered *in vivo* at a dosage of at least 0.1 ng/kg body weight or at a concentration of 10^{-11} to 10^{-7} M *in vitro* in cells with the natural androgen or estrogen receptor or transfected with the androgen or estrogen receptor; and (iv) has an anti-apoptotic effect on osteoblasts at an *in vivo* dosage of at least 0.1 ng/kg body weight or *in vitro* in cells with the natural androgen or estrogen receptor or transfected with the androgen or estrogen receptor.

Compounds selected according to the criteria provided herein can also be used as for the augmentation of bone mass and/or fracture prevention in diseases characterized by low bone mass and increased fragility. The compounds can be used to treat bone disease states in which osteoblastogenesis is decreased, such as senile osteoporosis, and glucocorticoid-induced osteoporosis--especially in growing children and adolescents, in whom interfering with bone remodeling is detrimental.

I. Definitions

An estrogen compound, as used herein, refers to a four ring steroidal compound which possesses the biological activity of an estrus-producing hormone, or its conjugated and esterified derivative, or a derivative thereof of same chemical composition

5 and structure but which does not possess the biological activity of
the active form because it exhibits a different stereochemistry
from the active form. Nonlimiting examples of estrogens include
10 broparestrol, chlorotrianisene, dienolestrol, epimestrol, equilin,
5 estrapronicate, estropipate, ethinylestradiol, fosfestrol,
hydroxyesetrone, mestranol, estradiol, estriol, conjugated and
15 esterified estrogens, estrone, polyestradiol, promestriene,
quinestradiol, quineestrol, stilbestrol, and zeranol.

An androgen compound, as used herein, refers to a
10 four ring steroidal compound which can be produced in the testis
or adrenal cortex, or is a synthetic hormone, which acts to regulate
masculine secondary sexual characteristics, or a derivative thereof
25 of same chemical composition and structure but which does not
possess the biological activity of the active form because it
15 exhibits a different stereochemistry from the active form.
Nonlimiting examples include boldenone, clostebol, danazol,
30 drosstanolone, epitioestanol, ethylestrenol, fluoxymesterone,
formebolone, furazabol, mepitioestane, mesterolone,
methandienone, methenolone, methyltestosterone, nandrolone,
35 norethandrolone, oxabolone, oxymetholone, prasterone,
20 quinbolone, stanolone, stanozolol, testosterone, and trenbolone.

As known, estrogens and androgens have chiral
40 carbons, and thus can exist in a number of stereochemical
configurations. Typically, for example, the 17β hydroxy estrogens
25 have biological activity while the 17α hydroxy estrogens have
45 very little effect on sexual characteristics (and induce little
hormone-like gene transcriptional activation). For the purpose of
this specification, any stereochemical configuration, including
50

5 either the biologically active or the biologically inactive or less
active structure, can be used, as long as the compound satisfies the
specifically itemized criteria of the invention.

10 The catalogue entitled "Steroids" from Steraloids Inc.,
5 Wilton, N.H., provides a list of over 3000 steroids, with numerous
estrogen and androgen derivatives. The catalog can be obtained
15 by contacting the company and is also currently available on the
internet at <http://www.steraloids.com>. One can select and
purchase compounds from this library, which are all commercially
20 available and thus easy to obtain and evaluate, for use in this
invention. One can also use known estrogen and androgen
receptor binding compounds.

25 The term "bone mass" refers to the mass of bone
mineral and is typically determined by Dual-Energy X-Ray
15 Absorbtiometry (DEXA).

30 The term "bone strength" refers to resistance to
mechanical forces and can be measured by any known method,
including vertebrae compression strength or three point -bending
of long bones.

35 20 The term "bone quality" refers to normal collagen
orientation without excessive accumulation of unmineralized bone
matrix, and can be measured by any known method, including
40 undecalcified bone histomorphometry.

45 25 The term "bone anti-resorption agent" refers to a
compound that blocks bone resorption by suppressing remodeling
or the activity and/or lifespan of osteoclasts.

50 The term "osteopenia" refers to decreased bone mass
below a threshold which compromises structural integrity.

As used herein, the terms "metabolic bone disease", "orthopedic bone disease" or "dental disease" are defined as conditions characterized by decreased bone mass and/or structural deterioration of the skeleton and/or teeth.

As used herein, the term "apoptosis" refers to programmed cell death characterized by nuclear fragmentation and cell shrinkage as detected by morphological criteria and Terminal Uridine Deoxynucleotidal Transferase Nick End Labeling (TUNEL) staining.

The term "host", as used herein, refers to any bone-containing animal, including, but not limited to humans, other mammals, canines, equines, felines, bovines (including chickens, turkeys, and other meat producing birds), cows, and bulls.

II. Compounds Useful in the Invention

A. Estrogen compounds that bind to the estrogen α or β receptor with an association constant of at least 10^8 M^{-1} , and preferably, at least 10^{10} M^{-1} , but which exhibit little transcriptional activation

According to the present invention, one can easily select estrogen compounds that significantly increase bone mass by evaluating them according to the disclosed criteria.

1. Binding to the estrogen α or β receptor

A compound should be selected that binds to the estrogen α or β receptor (or the equivalent receptor in the host animal) with an association constant of at least 10^8 M^{-1} , and preferably, at least 10^{10} M^{-1} . This constant can be measured by any known technique, including receptor binding assays whereby

5 ligand binding affinities are determined by competitive
radiometric binding assays using 10 nM [³H] estradiol as tracer,
10 purified estrogen receptor preparations, or cell cytosol
preparations, or intact cells, during one hour incubation at room
5 temperature or overnight at 4°. Bound receptor-ligand complex is
absorbed using hydroxylapatite.

15 The estrogen α and β receptor subtypes have
significantly different primary sequences in their ligand binding
and transactivation domains. ER α and ER β show a 56% amino acid
20 10 homology in the hormone binding domain/activation function-1
region, and only 20% homology in their A/B domain/activation
function-1 region. The difference between ER α and ER β structure
25 suggests that some compounds might bind ER α or ER β , but not
both. All such selectively binding compounds are considered to
15 fall within the scope of this invention.

30 Estrogen compounds include those described in the
11th Edition of "Steroids" from Steraloids Inc., Wilton, N. H., which
bind to the estrogen receptor with an association constant of at
least 10^8 M^{-1} , and preferably, at least 10^{10} M^{-1} .

35 20 2. Minimal effect on estrogen-induced transcriptional activation

40 In this embodiment, an estrogen compound is selected
that has a minimal effect on estrogen-induced transcriptional
activation (or suppression). The basis for this requirement is that
25 45 it has been discovered that apoptosis of osteoblasts is decreased
by receptor binding, in the absence of transcriptional activation
by estrogen-type compounds. Therefore, to provide a maximum
therapeutic efficacy on bone without causing unrelated and
50

5 undesired side estrogen-related effects, estrogen receptor ligands
with minimal transcriptional effects should be used.

10 To accomplish this separation of receptor binding and
transcriptional activity, a compound should be selected that
5 induces estrogenic gene transcriptional activity at a level that is
no greater than 10% that of 17 β -estradiol, and preferably no
15 greater than 5, 1 or even 0.1% that of 17 β -estradiol when
administered *in vivo* at a dosage of at least 0.1 ng/kg body weight
or *in vitro* in cells with natural estrogen receptors or transfected
20 with estrogen receptors or which induces an increase in uterine
weight of no more than 10% that of estrogen (or the equivalent
compound in a host animal).

25 One can determine whether a selected compound
induces estrogenic transcriptional activity at a level that is no
15 greater than 10% that of 17 β -estradiol, and preferably no greater
than 5, 1 or even 0.1% that of 17 β -estradiol when administered *in*
30 *vivo* at a dosage of at least 0.1 ng/kg body weight, by
administering the selected compound to a host, and then
35 monitoring the level of induction or suppression of a surrogate
20 marker of estrogenic transcriptional activity. Nonlimiting
examples of surrogate markers of estrogenic transcriptional
activation, include, but are not limited to, the expression of the
40 complement C-3 gene and lactoferin in the uterus.

In an alternative embodiment, the level of estrogen-
25 induced transcriptional activity can be assessed *in vitro*. One can
45 determine whether a selected compound induces transcriptional
activity at a level that is no greater than 10% that of 17 β -estradiol,
and preferably no greater than 5, 1 or even 0.1% that of 17 β -
50

5 estradiol *in vitro* using cells with natural estrogen receptors or
transfected with estrogen receptors, by monitoring the level of
induction or suppression of a surrogate marker. Nonlimiting
10 examples of genes induced or repressed by estrogen include, but
5 are not limited to, complement C-3, lactoferin, or interleukin-6. A
preferred marker gene for estrogenic transcriptional activity is a
minimal gene containing one or more copies of the ERE driving a
15 reporter gene such as luciferase.

Examples of cell lines that can be used include human
10 uterine HeLa cells, human embryonic kidney cells 293, murine
osteocytic MLO-Y4 cells and murine osteoblastic calvaria derived
cells.

One can assess the increase in uterine weight after
25 administration of the selected compound *in vivo*. Preferred
15 compounds induce an increase in uterine weight of no more than
approximately 10% that of estrogen (or the equivalent compound
30 in a host animal). This can be easily tested according to known
protocols. For example, in experimental mice, uteri are removed
and cleaned of adjacent ligaments and fat. Wet weight is
35 determined on a Mettler PB303 microgram balance (Toledo) and
20 compared to total body weight (mg/100g BW) as an index of the
estrogenic status of the animals. In women, similar assessment can
40 be performed by uterine ultrasound.

Examples of estrogen compounds that do not induce
25 significant estrogen-like transcriptional activity include, but are
45 not limited to estratriene-3-ol, 17 α -estradiol, 17 β -estradiol
conjugated with BSA.

**3. Induction of the phosphorylation of
extracellular signal regulated kinase (ERK)**

The selected compound should induce the phosphorylation of ERKs at a concentration of 10^{-11} to 10^{-7} M *in vitro* in cells with natural estrogen receptors or transfected with estrogen receptors using any known method, including but not limited to, the method set out in Figures 8 and 9 and Examples 7-9.

The phosphorylation of ERKs is easily assessed *in vitro* using osteoblastic or osteocytic cells with natural estrogen receptors or cells transfected with estrogen receptors. Examples of the evaluation of the phosphorylation of ERK in MLO-Y4 cells are provided in Figures 8 and 9 and Examples 7-9. Other appropriate cell models include osteoblastic cells isolated from neonatal murine calvaria.

4. Anti-apoptotic effect on osteoblasts at an *in vivo* dosage of at least 0.1 ng/kg body weight or at an *in vitro* concentration of 10^{-11} to 10^{-7} M or less.

The anti-apoptotic effect on osteoblasts *in vivo* can be assessed by any known method, including by the method described in Figure 2 and Example 3. The anti-apoptotic effect *in vitro* can be assessed by any known method including the methods described in Figures 3-7 and 10, and Examples 2-6 and 9.

B. Nonestrogen compounds that bind to the estrogen α or β receptor with an association constant

5 of at least 10^8 M^{-1} and preferably, at least 10^{10} M^{-1} ,
but which exhibit little transcriptional activation

10 1. Nonestrogen compound which binds to the
5 estrogen α or β receptor

A nonestrogen compound, as used herein, refers to a
15 compound other than an estrogen, as that term is defined above,
which binds to the estrogen α or β receptor with an association
constant of at least 10^8 M^{-1} and preferably, at least 10^{10} M^{-1} .

20 10 There are a number of reported compounds which are not
estrogens but which bind to the estrogen receptor.

Examples include the aryl-substituted pyrazole
25 described by Sun et al., Novel Ligands that Function as Selective
Estrogens or Antiestrogens for Estrogen Receptor- α or Estrogen
15 Receptor- β , Endocrinology, Volume 140, No. 2 (1999), one example
of which is illustrated below.

In an alternative embodiment, an estrogen or
nonestrogen compound is covalently linked to a second moiety
35 that does not significantly interfere with the binding to the
20 estrogen receptor but which does substantially prevent the
estrogen from entering the cell. In one example, the second
moiety is a protein such as bovine serum albumin, polyethelene
40 glycol or dextran or liposomes. In another embodiment, the
second moiety is not a protein or peptide, but for polar, steric, or
25 other reasons, prevents cell penetration. Examples of these types
of moieties include carboxylate, ammonium, and sulfide. A
45 "linking moiety" as used herein, is any divalent group that links
two chemical residues, including but not limited to alkyl, alkenyl,
50

alkynyl, aryl, polyalkyleneoxy (for example, $-\text{[(CH}_2\text{)}_n\text{O-]}_n-$), $-\text{C}_{1-6}\text{alkoxy-C}_{1-10}\text{alkyl-}$, $-\text{C}_{1-6}\text{alkylthio-C}_{1-10}\text{alkyl-}$, $-\text{NR}_3-$, and $-(\text{CHOH})_n\text{CH}_2\text{OH}$, wherein n is independently 0, 1, 2, 3, 4, 5, or 6, which can be attached at either end of the linking moiety to the structures of interest by any suitable functional groups. In an alternative embodiment, the linking moiety can be a bifunctional linker moiety of the formula $\text{X-(CH}_2\text{)}_n\text{-Y}$, wherein X and Y are functional groups capable of linking, including those independently selected from the group consisting of hydroxyl, sulfhydryl, carboxyl and amine groups, and n can be any integer between one and twenty four.

C. Androgen compounds that bind to the androgen receptor with an association constant of at least 10^8 M^{-1} , and preferably, at least 10^{10} M^{-1} , but which exhibit little transcriptional activation

According to the present invention, one can also easily select androgenic compounds that significantly increase bone mass by evaluating them according to the disclosed criteria.

1. Binding to the androgen receptor

A compound should be selected that binds to the androgen receptor (or the equivalent receptor in the host animal) with an association constant of at least 10^8 M^{-1} , and preferably, at least 10^{10} M^{-1} . The androgen receptor binding association constant is defined as the concentration of the ligand capable of saturating 50% of the unoccupied receptors. This constant can be measured by any known technique, including receptor binding assays

5 whereby ligand binding affinities are determined by competitive
radiometric binding assays using 10 nM [³H] of the synthetic
androgen RU1881 as tracer, purified androgen receptor
10 preparations, or cell cytosol preparations, or intact cells, during
5 one hour incubation at room temperature or overnight at 4°C.
Bound receptor-ligand complex is absorbed using hydroxylapatite.
15 Androgen compounds include those described in the 11th Edition
of "Steroids" from Steraloids Inc., Wilton, N.H., which bind to the
androgen receptor with an association constant of at least 10⁸ M⁻¹,
20 10 and preferably, at least 10¹⁰ M⁻¹.

2. Minimal effect on androgen-induced transcriptional activation

25 In this embodiment, an androgen compound is selected
that has a minimal effect on androgen-induced transcriptional
15 activation. The basis for this requirement, is that it has been
discovered that apoptosis of osteoblasts is decreased by receptor
30 binding in the absence of transcriptional activation by androgen-
type compounds. Therefore, to provide a maximum therapeutic
efficacy on bone without causing unrelated and undesired
35 20 androgen-related effects, androgen receptor ligands with minimal
transcriptional activity should be used.

40 To accomplish this separation of receptor binding and
transcriptional activity, a compound should be selected that
induces androgenic transcriptional activity at a level that is no
25 greater than 10% that of testosterone, and preferably no greater
45 than 5, 1 or even 0.1% that of testosterone when administered *in*
vivo at a dosage of at least 0.1 ng/kg body weight or *in vitro* in
cells with natural androgen receptors or transfected with
50

5 androgen receptors or induces an increase in prostate specific antigen (PSA) prostatic serum androgen of no more than 10% that of testosterone (or the equivalent compound in a host animal).

10 One can determine whether a selected compound
5 induces androgenic gene transcriptional activity at a level that is no greater than 10% that of testosterone, and preferably no
15 greater than 5, 1 or even 0.1% that of testosterone when administered *in vivo* at a dosage of at least 0.1 ng/kg body weight, by administering the selected compound to a host, and then
20 monitoring the level of induction or suppression of a surrogate marker of androgenic transcriptional activity. Nonlimiting examples of surrogate markers of androgenic transcriptional activation, include, but are not limited to prostate specific antigen (PSA).

15 In an alternative embodiment, the level of androgen-induced transcriptional activity can be assessed *in vitro* in
30 osteoblastic or osteocytic cells with natural androgen receptors or traf calvaria cells, MLO-Y4 osteocytic cells and HeLa cells.

35 Alternatively, one can assess the increase in PSA
20 serum levels after administration of the selected compound. Appropriate compounds induce an increase in PSA cells transfected with androgen receptors. Examples of such cell types
40 include, primary cultures of PSA of no more than approximately 10% that of testosterone (or the equivalent compound in a host
25 animal). This can be easily tested according to known protocols.

45 Examples of androgenic compounds that do not induce significant androgenic-like transcriptional activity include, but are

not limited to, testosterone 17 β -hemisuccinate conjugated with BSA.

3. Induction of the phosphorylation of extracellular signal regulated kinase (ERK)

The selected compound should induce the phosphorylation of ERKs when administered *in vivo* at a dosage of at least 0.1 ng/kg body weight or at a concentration of 10⁻¹¹ to 10⁻⁷ M *in vitro* in cells with natural androgenic receptors or transfected with androgenic receptors.

The phosphorylation of ERK in a host can be assessed in biopsies, for example from bone, using immunohistostaining with specific antibodies against phosphorylated ERKs. Alternatively, the phosphorylation of ERK is also easily assessed *in vitro* using osteoblastic or osteocytic cells with natural androgen receptors or cells transfected with androgen receptors. Examples of the evaluation of the phosphorylation of ERK in MLO-Y4 cells are provided Figures 8 and 9 and Examples 7-9.

4. Anti-apoptotic effect on osteoblasts and osteocytes at an *in vivo* dosage of at least 0.1 ng/kg body weight or at an *in vitro* concentration of 10⁻¹¹ to 10⁻⁷ M or less.

The anti-apoptotic effect on osteoblasts and osteocytes can be assessed *in vivo* by any known method, including the method described in Figure 2 and Example 1; and *in vitro* by any known method, including the method described in Figures 3-7 and 10 and Examples 2-6 and 9.

5 **D. Nonandrogen compounds that bind to th**
 androgen receptor with an association constant of at
 least 10^8 M^{-1} , and preferably, at least 10^{10} M^{-1} , but
10 **which exhibit little transcriptional activation**

5

 A nonandrogenic compound, as used herein, refers to a
15 compound other than an androgen, as that term is defined above,
 which binds to the androgenic receptor with an association
 constant of at least 10^8 M^{-1} and preferably, at least 10^{10} M^{-1} .

10 There are a number of reported compounds which are not
20 androgens but which bind to the androgen receptor. Examples
 include testosterone 17 β -hemisuccinate conjugated with BSA.

25 In an alternative embodiment, an androgen compound
 is covalently linked to a second moiety that does not significantly
15 interfere with the binding to the androgen receptor but which
 does substantially prevent the androgen from entering the cell. In
30 one example, the second moiety is a protein such as bovine serum
 albumin. In another embodiment, the second moiety is not a
 protein or peptide, but for polar, steric, or other reasons, prevents
35 cell penetration. Examples of these types of moieties include
20 dextran or polyethylene glycol.

40 **E. Other compounds that can be used to increase**
 bone mass.

 Other nonlimiting examples of compounds that can be
25 used in the present invention to increase bone mass include those
45 having a terminal phenyl ring and at least a second carbon ring.
 In addition to these required structures, the compound may have
 a number of R groups attached to any available site on the phenyl
50

5 ring or elsewhere. These R groups may be selected from inorganic
or organic atoms or moieties. Representative R groups are
provided, although the invention is not to be limited by these
10 examples:

- 5 (a) The R₁ or R₂ groups may include a hydroxyl
group or an inorganic R group including any of a halogen, an
15 amide, a sulfate, a nitrate, fluoro, chloro, or bromo groups.
Additionally, R₁ or R₂ groups such as sodium, potassium and/or
ammonium salts may be attached to the alpha or beta positions to
20 replace hydrogen on any available carbon in the structure. The R₁
or R₂ groups may be organic or may include a mixture of organic
molecules and ions. Organic R₁ or R₂ groups may include alkanes,
25 alkenes or alkynes containing up to six carbons in a linear or
branched array. For example, additional R₁ or R₂ group
15 substituents may include methyl, ethyl, propyl, butyl, pentyl,
hexyl, heptyl, dimethyl, isobutyl, isopentyl, tert-butyl, sec-butyl,
30 isobutyl, methylpentyl, neopentyl, isohexyl, hexenyl, hexadiene,
1,3-hexadiene-5-yne, vinyl, allyl, isopropenyl, ethynyl, ethylidene,
vinylidene, isopropylidene, methylene, sulfate, mercapto,
35 methylthio, ethylthio, propylthio, methylsulfinyl, methylsulfonyl,
20 thiohexanyl, thiobenzyl, thiophenol, thicyanato, sulfoethylamide,
thionitrosyl, thiophosphoryl, p-toluenesulfonate, amino, imino,
40 cyano, carbamoyl, acetamido, hydroxyamino, nitroso, nitro,
cyanato, selecyanato, arccosine, pyridinium, hydrazide,
25 semicarbazone, carboxymethylamide, oxime, hydrazone,
45 sulfurtrimethylammonium, semicarbazone, o-
carboxymethyloxime, aldehyde hemiacetate, methylether,
ethylether, propylether, butylether, benzylether, methylcarbonate,
50

5 carboxylate, acetate, chloroacetate, trimethylacetate,
cyclopentylpropionate, propionate, phenylpropionate, carboxylic
acid methylether, formate, benzoate, butyrate, caprylate,
10 cinnamate, decylate, heptylate, enanthate, glucosiduronate,
5 succinate, hemisuccinate, palmitate, nonanoate, stearate, tosylate,
valerate, valproate, decanoate, hexahydrobenzoate, laurate,
15 myristate, phthalate, hydroxyl, ethyleneketal, diethyleneketal,
formate, chloroformate, formyl, dichloroacetate, keto,
difluoroacetate, ethoxycarbonyl, trichloroformate,
10 hydroxymethylene, epoxy, peroxy, dimethyl ketal, acetone,
cyclohexyl, benzyl, phenyl, diphenyl, benzylidene, and cyclopropyl
groups. R₁ or R₂ groups may be attached to any of the constituent
rings to form a pyridine, pyrazine, pyrimidine, or v-triazine.
25 Additional R₁ or R₂ group substituents may include any of the six-
15 member or five-member rings itemized in section (b) below.

(b) Any compound having, in addition to the
30 terminal phenyl group, at least one heterocyclic carbon ring
(shown as R₃ in Figure 1), which may be an aromatic or non-
aromatic phenolic ring with any of the substitutions described in
35 section (a) above, and further may be, for example, one or more of
20 the following structures: phenanthrene, naphthalene, naphthols,
diphenyl, benzene, cyclohexane, 1,2-pyran, 1,4-pyran, 1,2-pyrone,
40 1,4-pyrone, 1,2-dioxin, 1,3-dioxin (dihydro form), pyridine,
pyridazine, pyrimidine, pyrazine, piperazine, s-triazine, as-
25 triazine, v-triazine, 1,2,4-oxazine, 1,3,2-oxazine, 1,3,6-oxazine
45 (pentoxazole), 1,2,6-oxazine, 1,4-oxazine, o-isoxazine, p-isoxazine,
1,2,5-oxathiazine, 1,2,6-oxathiazine, 1,4,2-oxadiazine, 1,3,5,2-
oxadiazine, morpholine (tetrahydro-p-isoxazine), any of the six-
50

5 ringed structures listed above being a terminal group in the
compound. Additionally, any of the above carbon ring structure
may be linked directly, or via a linkage group, to any further
10 heterocyclic aromatic or non aromatic carbon ring including: furan,
5 thiophene (thiofuran), pyrrole (azole), isopyrrole (isoazole), 3-
isopyrrole (isoazole), pyrazole (1,2 diazole), 2-isoimidazole (1,3-
isodiazole), 1,2,3-triazole, 1,2,4-triazole, 1,2-dithiazole, 1,2,3-
15 oxathiazole, isoxazole (furo(a) monozole), oxazole (furo(b)
monazole), thiazole, isothiazole, 1,2,3-oxathiazole, 1,2,4-oxadiazole,
20 1,2,5-oxadiazole, 1,3,5-oxadiazole, 1,2,3,4-oxatriazole, 1,2,3,5-
oxatriazole, 1,2,3-dioxazole, 1,2,4-dioxazole, 1,3,2-dioxazole, 1,3,4-
dioxazole, 1,2,5-oxathiazole, 1,3-oxathiazole, cyclopentane. These
25 compounds, in turn, may have associated R₁ or R₂ groups selected
from section (a) or section (b) above that are substituted on the
15 carbon ring at any of the available sites.

30 (c) Any compound, including those listed above, that
may form a cyclopentanophen(a)anthrene ring compound and
which, for example, may be selected from the group consisting of
1,3,5(10),6,8-estrapentaene, 1,3,5(10),6,8,11-estrapentaene,
35 20 1,3,5(10),6,8,15-estrapentaene, 1,3,5(10),6-estratetraene,
1,3,5(10),7-estratetraene, 1,3,5(10),8-estratetraene, 1,3,5(10),16-
estratetraene, 1,3,5(10),15-estratetraene, 1,3,5(10)-estratriene,
40 1,3,5(10),15-estratriene.

25 (d) Any compound including precursors or
derivatives selected from raloxifen, tamoxifen, androgenic
45 compounds, and their salts, where an intact phenol ring is present
with a hydroxyl group present on carbons 1, 2, 3 and 4 of the
terminal phenol ring.
50

5 (e) Any compound in the form of a prodrug that
may be metabolized to form an active polycyclic-phenolic
compound having bone protective activity.

10 **III. Methods for Using the Active Compounds**

5 The active compounds which satisfy the criteria set out
in detail herein can be used to treat a wide variety of medical
15 conditions, including any condition in which it is helpful or
necessary to build bone mass. Because of the discovery of the
fundamental basis for bone loss (inappropriate osteoblastic
20 apoptosis), one can for the first time envision the building of
healthy bone as opposed to merely treating bone loss.

The active compounds can be used as bone anabolic
25 agents in a host, including a human, to strengthen bone for
strenuous physical activities such as sports or manual labor, and
15 to strengthen bone in persons or other hosts who do not have
osteoporosis but might be subject to osteoporosis in the future
30 because the host is in a risk group for that disease. Other uses for
a bone anabolic agent in humans include the treatment of hosts,
including persons who are born with naturally thin, small, or
35 20 unusually fragile bones, including weak teeth, persons who have a
genetic predisposition to a bone catabolic disease, or an orthopedic
bone disease such as joint degeneration, non-union fractures,
40 orthopedic problems caused by diabetes, periimplantitis, poor
responses to bone grafts, implants, or fracture.

25 These compounds can be used to increase the bone
45 mass in horses and dogs used for labor as well as those used in
sports such as racing. The compounds can also be used to increase

5 the bone mass in chickens and turkeys used in meat production to increase the ease of processing.

10 Representative metabolic bone diseases are postmenopausal osteoporosis, senile osteoporosis in males and
5 females, glucocorticoid-induced osteoporosis, immobilization-induced osteoporosis, weightlessness-induced osteoporosis (as in
15 space flights), post-transplantation osteoporosis, migratory osteoporosis, idiopathic osteoporosis, juvenile osteoporosis, Paget's Disease, osteogenesis imperfecta, chronic hyperparathyroidism,
20 hyperthyroidism, rheumatoid arthritis, Gorham-Stout disease, McCune-Albright syndrome and osteolytic metastases of various cancers or multiple myeloma. Characteristics of the orthopedic bone diseases are loss of bone mass, general bone fragility, joint degeneration, non-union fractures, orthopedic and dental
25 problems caused by diabetes, periimplantitis, poor responses to bone grafts/implants/bone substitute materials, periodontal diseases, and skeletal aging and its consequences.

30 IV. Method for Screening for Compounds that Increase Bone Mass

35 20 The present invention provides a method of screening for compounds that possess bone anabolic effects, comprising the steps of: a) contacting a sample of osteoblast cells with a
40 compound; and b) comparing the number of osteoblast cells undergoing apoptosis in the compound-treated cells with the
25 number of osteoblast cells undergoing apoptosis in an untreated sample of osteoblast cells. A lower number of apoptotic cells
45 following contact with the compound indicates that the compound possesses bone anabolic effects. Preferred compounds also inhibit
50

5 apoptosis of osteocytes. Generally, the compound may be
contacted with the sample either *in vitro*, e.g., in cell culture or *in*
10 *vivo*, e.g., in an animal model. Typical methods of determining
apoptosis are nuclear morphologic criteria, DNA end-labeling, DNA
5 fragmentation analysis and immunohistochemical analysis.

In another embodiment, a method for selecting a
15 compound that increases bone mass at least 10% in a host without
a loss in bone strength or quality is provided that includes
evaluating whether the compound (i) binds to the estrogen or
20 androgen receptor (or the equivalent receptor in the host animal)
with an association constant of at least 10^8 M^{-1} , and preferably, at
least 10^{10} M^{-1} ; (ii) (a) induces estrogenic or androgenic gene
transcriptional activity at a level that is no greater than 10% that
25 of testosterone or 17β -estradiol, and preferably no greater than 5,
15 1 or even 0.1% that of 17β -estradiol or testosterone, as
appropriate, when administered *in vivo* at a dosage of at least 0.1
30 ng/kg body weight or *in vitro* at concentrations of 10^{-11} to 10^{-7} M
in cells with the natural androgen or estrogen receptor or
transfected with the androgen or estrogen receptor or (b) induces
35 20 an increase in uterine or muscle weight or increase virilization in
females, as appropriate, of no more than 10% that which is
induced by 17β -estradiol or testosterone (or the equivalent
40 compound in a host animal); (iii) induces the phosphorylation of
extracellular signal regulated kinase (ERK) when administered *in*
25 *vivo* at a dosage of at least 0.1 ng/kg body weight or *in vitro* in
45 cells with the natural androgen or estrogen receptor or transfected
with the androgen or estrogen receptor; and (iv) has an anti-
50 apoptotic effect on osteoblasts at an *in vivo* dosage of at least 0.1

5 ng/kg body weight or *in vitro* in cells with the natural androgen or estrogen receptor or transfected with the androgen or estrogen receptor.

10 In another embodiment, a method for screening for
5 compounds that bind to the estrogen or androgen receptor and activate the anti-apoptotic signalling pathway, without resultant transcriptional activation, is provided. This method is based on
15 the fundamental discovery that the ligand-induced conformational changes of the estrogen receptor protein required for prevention
20 of apoptosis, are distinct from the conformational changes required for transcriptional activity (Figures 19-21). This discovery allows for selecting compounds, from a large library of
25 small molecules, which have anti-apoptotic, but not transcriptional, activity. Selection is accomplished using small
15 peptides that can specifically block the transcriptional activity of ligand activated receptor, but do not interfere with the ability of
30 the receptor to initiate the anti-apoptotic signalling cascade.

To accomplish this, cells are transfected with the
35 estrogen or androgen receptor with or without a peptide that
20 recognizes the conformation of the protein required for transcriptional activation, but not anti-apoptosis. Using this method, compounds that induce conformational changes resulting
40 in both transcriptional and anti-apoptosis compatible conformations can be distinguished from compounds that only
25 induce the latter conformational changes.

45 Nonlimiting examples of this method of screening include peptide binding assays for ER α or ER β whereby the purified receptor protein is immobilized on streptavidin-coated
50

5 plates using biotinylated vitellogenin ERE according to previously
described methods of affinity selection (Sparks AB, Adey NB,
Cwirla S, Kay BK. Screening phage-displayed peptide libraries. In
10 *Phage Display of Peptides and Proteins, A Laboratory Manual*, eds.
5 Kay BK, Winter J and McCafferty J. (Academic, San Diego), pp.227-
253, 1996). Following incubation with various ligands, the peptide
15 is added and after 30 min bound peptide is detected using an
anti-M13 antibody coupled to horseradish peroxidase. Compounds
that bind to the receptor and induce conformational changes
20 10 recognized by the peptide (i.e. the peptide binds to the receptor)
will be discarded. The remaining compounds are then screened
for anti-apoptotic potency.

25 V. Combination Therapy

In one aspect of the invention, one of the active
15 compounds described herein can be administered to a host to
increase bone mass in combination with a second pharmaceutical
30 agent. The second pharmaceutical agent can be a bone anti-
resorption agent, a second bone mass anabolizing agent, an
antioxidant, a dietary supplement, or any other agent that
35 20 increases the beneficial effect of the active compound on bone
structure, strength, density, or mass.

Any member of the ten classes of drugs described in
40 the Background of the Invention that are used in the treatment of
osteoporosis can be administered in combination with the primary
25 45 active agent, including: an anabolic steroid, a bisphosphonate, a
calcitonin, an estrogen or progesterone, an anti-estrogens such as
raloxifene or tamoxifene, parathyroid hormone ("PTH"), fluoride,
Vitamin D or a derivative thereof, or a calcium preparations.

5 Nonlimiting examples of suitable agents for
combination include, but are not limited to, alendronic acid,
disodium clondronate, disodium etidronate, disodium medronate,
10 disodium oxidronate, disodium pamidronate, neridronic acid,
5 risedronic acid, teriparatide acetate, tiludronic acid, ipriflavone,
potassium bicarbonate, progestogen, a thiazide, gallium nitrate,
15 NSAIDS, plicamycin, aluminum hydroxide, calcium acetate, calcium
carbonate, calcium, magnesium carbonate, and sucralfate.

Reducing agents, such as glutathione or other
10 antioxidants may also be useful in combination with any of the
compounds of the present invention. As used herein, the term
antioxidant refers to a substance that prevents the oxidation of an
oxidizable compound under physiological conditions. In one
25 embodiment, a compound is considered an antioxidant for
15 purposes of this disclosure if it reduces endogenous oxygen
radicals *in vitro*. The antioxidant can be added to a cell extract
under oxygenated conditions and the effect on an oxidizable
30 compound evaluated. As nonlimiting examples, antioxidants
scavenge oxygen, superoxide anions, hydrogen peroxide,
35 superoxide radicals, lipooxide radicals, hydroxyl radicals, or bind
20 to reactive metals to prevent oxidation damage to lipids, proteins,
nucleic acids, etc. The term antioxidant includes, but is not limited
40 to, the following classes of compounds:

A) Dithiocarbamates: Dithiocarbamates have been
25 extensively described in patents and in scientific literature.
45 Dithiocarbamates and related compounds have been reviewed
extensively for example, by G. D. Thorn et al., entitled "The
Dithiocarbamates and Related Compounds," Elsevier, New York,
50

5 1962. Dithiocarboxylates are compounds of the structure, A-
SC(S)-B, which are members of the general class of compounds
known as thiol antioxidants, and are alternatively referred to as
10 carbodithiols or carbodithiolates. It appears that the -SC(S)-
5 moiety is essential for therapeutic activity, and that A and B can
be any group that does not adversely affect the efficacy or toxicity
of the compound. A and B can be selected by one of ordinary skill
15 in the art to impart desired characteristics to the compound,
including size, charge, toxicity, and degree of stability, (including
20 stability in an acidic environment such as the stomach, or basic
environment such as the intestinal tract). The selection of A and B
will also have an important effect on the tissue-distribution and
25 pharmacokinetics of the compound. The compounds are
preferably eliminated by renal excretion.

15 B) N-Acetyl Cysteine and its Derivatives

30 Cysteine is an amino acid with one chiral carbon atom.
It exists as an L-enantiomer, a D-enantiomer, or a racemic mixture
of the L- and D-enantiomers. The L-enantiomer is the naturally
occurring configuration.

35 20 N-acetylcysteine (acetamido-mercaptopropionic acid,
NAC) is the N-acetylated derivative of cysteine. It also exists as an
L-enantiomer, a D-enantiomer, an enantiomerically enriched
40 composition of one of the enantiomers, or a racemic mixture of the
L and D enantiomers. The term "enantiomerically enriched
25 composition or compound" refers to a composition or compound
that includes at least 95%, and preferably, at least 97% by weight
45 of a single enantiomer of the compound. Any of these forms of
NAC can be delivered as an antioxidant in the present invention.

5 In one embodiment, a single isomer of a thioester or thioether of
NAC or its salt, and most preferably, the naturally occurring L-
enantiomer, is used in the treatment process.

10 N-acetylcysteine exhibits antioxidant activity
5 (Smilkstein, Knapp, Kulig and Rumack, *N. Engl. J. Med.* 1988, Vol.
319, pp. 1557-62; Knight, K.R., MacPhadyen, K., Lepore, D.A.,
15 Kuwata, N., Eadie, P.A., O'Brien, B. *Clinical Sci.*, 1991, Vol. 81, pp.
31-36; Ellis, E.F., Dodson, L.Y., Police, R.J., *J. Neurosurg.*, 1991, Vol.
75, pp. 774-779). The sulfhydryl functional group is a well
20 characterized, highly reactive free radical scavenger. N-
acetylcysteine is known to promote the formation of glutathione (a
tri-peptide, also known as g-glutamylcysteinylglycine), which is
25 important in maintaining cellular constituents in the reduced state
(Berggren, M., Dawson, J., Moldeus, P. *FEBS Lett.*, 1984, Vol. 176,
15 pp. 189-192). The formation of glutathione may enhance the
activity of glutathione peroxidase, an enzyme which inactivates
30 hydrogen peroxide, a known precursor to hydroxyl radicals
(Lalitha, T., Kerem, D., Yanni, S., *Pharmacology and Toxicology*,
1990, Vol.66, pp. 56-61)

35 20 N-acetylcysteine exhibits low toxicity *in vivo*, and is
significantly less toxic than deprenyl (for example, the LD₅₀ in rats
has been measured at 1140 and 81 mg/kg intravenously, for N-
40 acetylcysteine and deprenyl, respectively).

N-acetyl cysteine and derivatives thereof are
25 described, for example, in WO/95/26719. Any of the derivatives
45 described in this publication can be used in accordance with this
invention.

5 C) Scavengers of Peroxides, including but not limited to catalase and pyruvate.

D) Thiols including dithiothreitol and 2-mercaptoethanol.

10 E) Antioxidants which are inhibitors of lipid peroxidation, including but not limited to TroloxTM, BHA, BHT, aminosteroid antioxidants, tocopherol and its analogs, and
15 lazaroids.

F) Dietary antioxidants, including antioxidant vitamins (vitamin C or E or synthetic or natural prodrugs or analogs
20 thereof), either alone or in combination with each other, flavanoids, phenolic compounds, caratenoids, and alpha lipoic acid.

G) Inhibitors of lipoxygenases and cyclooxygenases, including but not limited to nonsteriodal antiinflammatory drugs,
25 COX-2 inhibitors, aspirin-based compounds, and quercetin.

15 H) Antioxidants manufactured by the body, including but not limited to ubiquinol and thiol antioxidants, such as, and
30 including glutathione, Se, and lipoic acid.

I) Synthetic Phenolic Antioxidants: inducers of Phase I and II drug-metabolizing enzymes.

35 20 **VI. Pharmaceutical Compositions**

An active compound or its pharmaceutically acceptable salt, selected according to the criteria described in detail herein,
40 can be administered in an effective amount to treat any of the conditions described herein, optionally in a pharmaceutically
25 acceptable carrier or diluent.

45 The active materials can be administered by any appropriate route for systemic, local or topical delivery, for example, orally, parenterally, intravenously, intradermally,
50

5 subcutaneously, buccal, intranasal, inhalation, vaginal, rectal or
topically, in liquid or solid form. Methods of administering the
compound of the invention may be by specific dose or by
10 controlled release vehicles.

5 A preferred mode of administration of the active
compound is oral. Oral compositions will generally include an
inert diluent or an edible carrier. The active compound can be
15 enclosed in gelatin capsules or compressed into tablets. For the
purpose of oral therapeutic administration, the compound can be
20 incorporated with excipients and used in the form of tablets,
troches, or capsules. Pharmaceutically compatible binding agents,
and/or adjuvant materials can be included as part of the
25 composition.

The tablets, pills, capsules, troches and the like can
15 contain any of the following ingredients, or compounds of a similar
nature: a binder such as microcrystalline cellulose, gum tragacanth
or gelatin; an excipient such as starch or lactose, a disintegrating
agent such as alginic acid, Primogel, or corn starch; a lubricant
such as magnesium stearate or Sterotes; a glidant such as colloidal
35 silicon dioxide; a sweetening agent such as sucrose or saccharin;
20 and/or a flavoring agent such as peppermint, methyl salicylate, or
orange flavoring. When the dosage unit form is a capsule, it can
contain, in addition to material of the above type, a liquid carrier
such as a fatty oil. In addition, dosage unit forms can contain
40 various other materials which modify the physical form of the
dosage unit, for example, coatings of sugar, shellac, or other
25 enteric agents.

5 The compound can be administered as a component of
an elixir, suspension, syrup, wafer, chewing gum or the like. A
syrup may contain, in addition to the active compounds, sucrose as
10 a sweetening agent and certain preservatives, dyes and colorings
5 and flavors.

 The compound or a pharmaceutically acceptable
15 derivative or salts thereof can also be mixed with other active
materials that do not impair the desired action, or with materials
that supplement the desired action, such as classical estrogen like
20 17 β -estradiol or ethinyl estradiol; bisphosphonates like
alendronate, etidronate, pamidronate, risedronate, tiludronate,
zoledronate, cimadronate, clodronate, ibandronate, olpadronate,
25 neridronate, EB-1053; calcitonin of salmon, eel or human origin;
and anti-oxidants like glutathione, ascorbic acid or sodium
15 bisulfite. Solutions or suspensions used for parenteral,
intradermal, subcutaneous, or topical application can include the
30 following components: a sterile diluent such as water for injection,
saline solution, fixed oils, polyethylene glycols, glycerine,
35 propylene glycol or other synthetic solvents; antibacterial agents
20 such as benzyl alcohol or methyl parabens; chelating agents such
as ethylenediaminetetraacetic acid (EDTA); buffers such as
40 acetates, citrates or phosphates and agents for the adjustment of
tonicity such as sodium chloride or dextrose. The parental
preparation can be enclosed in ampoules, disposable syringes or
25 multiple dose vials made of glass or plastic. If administered
45 intravenously, preferred carriers are physiological saline or
phosphate buffered saline (PBS).

5 In a preferred embodiment, the active compounds are
prepared with carriers that will protect the compound against
rapid elimination from the body, such as a controlled release
10 formulation, including implants and microencapsulated delivery
5 systems. Biodegradable, biocompatible polymers can be used,
such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid,
collagen, polyorthoesters, and polylactic acid. Methods for
15 preparation of such formulations will be apparent to those skilled
in the art.

20 10 Liposomal suspensions (including liposomes targeted
with monoclonal antibodies to surface antigens of specific cells)
are also pharmaceutically acceptable carriers. These may be
prepared according to methods known to those skilled in the art,
25 for example, as described in U.S. Patent No. 4,522,811 (which is
15 incorporated herein by reference in its entirety). For example,
liposome formulations may be prepared by dissolving appropriate
lipid(s) (such as stearyl phosphatidyl ethanolamine, stearyl
phosphatidyl choline, arachadyl phosphatidyl choline, and/or
30 cholesterol) in an inorganic solvent that is then evaporated,
35 leaving behind a thin film of dried lipid on the surface of the
20 container. An aqueous solution of the active compound or its
monophosphate, diphosphate, and/or triphosphate derivative(s) is
40 then introduced into the container. The container is then swirled
by hand to free lipid material from the sides of the container and
25 to disperse lipid aggregates, thereby forming the liposomal
45 suspension.

The dose and dosage regimen will depend upon the
50 nature of the metabolic bone disease, the characteristics of the

5 particular active compound, *e.g.*, its therapeutic index, the patient,
the patient's history and other factors. The amount of an activator
of non-genomic estrogen-like signaling compound administered
10 will typically be in the range of about 1 pg/kg to about 10 mg/kg
5 of patient weight. The schedule will be continued to optimize
effectiveness while balanced against negative effects of treatment.
15 See Remington's Pharmaceutical Science, 17th Ed. (1990) Mark
Publishing Co., Easton, Penn.; and Goodman and Gilman's: The
Pharmacological Basis of Therapeutics 8th Ed (1990) Pergamon
20 10 Press.

For parenteral administration, the active compound
will most typically be formulated in a unit dosage injectable form
25 (solution, suspension, emulsion) in association with a
pharmaceutically acceptable parenteral vehicle. Such vehicles are
15 preferably non-toxic and non-therapeutic. Examples of such
vehicles are water, saline, Ringer's solution, dextrose solution, and
30 5% human serum albumin. Nonaqueous vehicles such as fixed oils
and ethyl oleate may also be used. Liposomes may be used as
carriers. The vehicle may contain minor amounts of additives
35 20 such as substances that enhance isotonicity and chemical stability,
e.g., buffers and preservatives. An activator of non-genomic
estrogen-like signaling compound will typically be formulated in
40 such vehicles at concentrations of about 10 pg/ml to about 10
mg/ml.

25 The concentration of the compound in the drug
45 composition will depend on absorption, inactivation, and excretion
rates of the drug as well as other factors known to those of skill in
the art. It is to be noted that dosage values will also vary with the
50

5 severity of the condition to be alleviated. Additionally, the active
ingredient may be administered at once, or may be divided into a
10 number of smaller doses to be administered at varying intervals
of time. It is to be further understood that for any particular
5 patient, specific dosage regimens should be adjusted over time
according to the individual need and the professional judgment of
15 the person administering or supervising the administration of the
compositions, and that the concentration ranges set forth herein
are exemplary only and are not intended to limit the scope or
20 practice of the claimed composition.

VII. Illustrative Examples

25 The following examples are illustrations of the
embodiments of the invention as described above, but are not
intended to limit its scope.

15 As one example, 17β -estradiol, the synthetic steroid
estratriene-3-ol, which is a potent neuroprotective compound, and
30 17α -estradiol, have potent anti-apoptotic effects on osteoblastic
cells *in vitro*.

35 U.S. Patent No. 5,843,934 to Simpkins discloses that an
20 estrogen having insubstantial sex-related activity, and in
particular, α -estrogens such as 17α -estradiol, can be administered
40 to a patient to retard the adverse effects of osteoporosis in a male
or female. The '934 patent does not address how to select a
compound to increase bone mass opposed to treat osteoporosis.
45 Increasing bone mass is a different indication from the treatment
of bone loss, as dramatically illustrated by the fact that the U.S.
Food and Drug Administration has approved a number of drugs

5 for the treatment of osteoporosis, but has not approved any drugs
to date as bone anabolic agents.

10 17 β -Estradiol is used in these illustrative examples
even though it is a potent activator of estrogen-like gene
5 transcription, because it tightly binds to the estrogen receptor and
inhibits osteoblastic apoptosis. The compound must be modified
15 to fall within the selection criteria for the present invention by
altering it in such a way that it cannot enter the cell to induce
gene transcription. Such modifications can occur, for example, by
20 10 covalently attaching, either directly or through a linking moiety, a
second moiety that prevents or limits cell penetration. Any other
estrogen or androgen that binds appropriately to the relevant
25 receptor can be likewise modified for use to increase bone mass.

It is noteworthy that (a) the anti-apoptotic effect of
15 17 β -estradiol on both osteoblasts and osteocytes are reproduced
with a membrane impermeable 17 β -estradiol - BSA conjugate; (b)
30 the anti-apoptotic effects of these compounds are diminished by
ICI 182780, a pure estrogen receptor antagonist; and (c) that the
35 anti-apoptotic effects of all these compounds cannot be shown in
20 HeLa cells unless these cells are stably transfected with either the
estrogen receptor α or the estrogen receptor β .

40 The following examples are given for the purpose of
illustrating various embodiments of the invention and are not
meant to limit the present invention in any fashion.

EXAMPLE 1

The increased rate of bone remodeling that follows loss of estrogen should cause a transient acceleration of mineral loss because bone resorption is faster than bone formation and the bone made by new BMUs are less dense than older ones. However, increased remodeling alone cannot explain the progressive bone loss that lasts long after the rate of bone remodeling has slowed. Indeed, in addition to changes in the number of osteoblast and osteoclast cells during/following estrogen deficiency, a qualitative abnormality also occurs; osteoclasts erode deeper than normal cavities. This frequently leads to penetration through a trabecular structure causing removal of some cancellous elements entirely; the remainder are more widely separated and less well connected. The deeper erosion is explained by loss of estrogen's effect to promote apoptosis of osteoclasts (Hughes et al, Nature Med. 1996; 2:1132-1136; Kameda et al, J Exp Med. 1997; 186:489-495; Raisz, Nature Med. 1996; 2:1077-1078). 17β -estradiol increased the apoptosis of osteoclasts from approximately 0.5% to as much as 2.7%. This change could prolong the lifespan of osteoclasts and increase their numbers two- to three-fold, thus accounting for the perforation of trabeculae and grinding away of endocortical margins.

To determine whether the role of estrogen deficiency affects osteoblast and osteocyte apoptosis, the prevalence of these cells in murine vertebrae removed 28 days after ovariectomy was determined. In these experiments, four month old Swiss Webster mice were ovariectomized and 28 days later, the animals were

5 sacrificed and the vertebrae were isolated, fixed and embedded
undecalcified in methacrylate. As shown in Figure 2, the
prevalence of osteoblast and osteocyte apoptosis, determined by
10 TUNEL with CuSO_4 enhancement, increased ten- and four-fold,
5 respectively. These results indicate that the accelerated loss of
bone that occurs after estrogen deficiency is due not only to an
increase in osteoclast number and lifespan, but also to a
15 premature reduction in the lifespan (work hours) of the
osteoblasts. The increase in osteocyte apoptosis could further
20 10 weaken the skeleton by impairment of the osteocyte-canalicular
mechanosensory network.

25 EXAMPLE 2

15 Consistent with the *in vivo* data described under
Example 1, 17β -estradiol prevented apoptosis of osteoblastic cells
30 isolated from murine calvaria, in a dose dependent manner.
Strikingly, inhibition of osteoblast apoptosis could also be shown
by 17β -estradiol conjugated with bovine serum albumin, a
35 20 membrane impermeable compound. The same effect could also be
shown with 17α -estradiol, a compound heretofore thought to be
inactive. Moreover, inhibition of etoposide-induced osteoblastic
40 cell apoptosis was demonstrated by estratriene-3-ol, an estrogenic
compound thought to lack feminizing properties (Figure 3). In this
25 45 experiment, osteoblastic cells were derived from murine calvaria
and were pretreated with the sterols for 1 hour before the
addition of the pro-apoptotic agent, etoposide.

EXAMPLE 3

In agreement with the *in vivo* results indicating that estrogen loss increases both osteoblast and osteocyte apoptosis, 17 β -estradiol, 17 β -estradiol conjugated with BSA, 17 α -estradiol, and estratriene-3-ol dose-dependently inhibited also the apoptosis of an established osteocytic cell line (Figure 4). In this experiment, MLO-Y4 cells were pretreated with the indicated concentrations of the various compounds for 1 h before the addition of the pro-apoptotic agent, etoposide. Apoptosis was determined after 6 h by trypan blue uptake as described in Figure 3.

EXAMPLE 4

As shown in Figure 5, the anti-apoptotic effect of 10⁻⁸ M 17 β -estradiol, 17 β -estradiol-BSA, 17 α -estradiol, or estratriene-3-ol (E-3-ol) on osteoblastic cells was abrogated when the cells were pretreated for 1 h with the pure receptor antagonist ICI182,780 (10⁻⁷ M) before the addition of the estrogenic compounds.

EXAMPLE 5

As in the case of the antiapoptotic effect of 17 β -estradiol, 17 β -estradiol-BSA, 17 α -estradiol, or estratriene-3-ol (E-3-ol) on osteoblastic cells, their antiapoptotic effect on osteocytes was abrogated when the cells were pretreated for 1 h with the

5 pure receptor antagonist ICI182,780 (10^{-7} M). Collectively, the
results of examples 4 and 5 strongly suggest that the anti-
apoptotic effects of these compounds on osteoblasts and osteocytes
10 are mediated via the estrogen receptor (ER).

5

EXAMPLE 6

15

Definitive demonstration of the requirement of the
estrogen receptor for the anti-apoptotic effects of 17β -estradiol
10 and the related compounds tested herein was provided by the
results of the experiment shown in Figure 7. In this experiment,
instead of calvaria cells, human HeLa cells which contain
undetectable, if any, estrogen receptor were used. HeLa cells were
25 stably transfected with either a CMV promoter-driven cDNA for
the murine estrogen receptor-alpha ($mER\alpha$) or a CMV promoter-
15 driven cDNA for the murine estrogen receptor-beta ($mER\beta$).
Subconfluent cultures of stable transfectants were treated for 1 h
with 17β -estradiol, or 17α -estradiol, estratriene-3-ol (10^{-8} M),
30 followed by a 6 hour incubation with etoposide (5×10^{-5} M). Cells
35 were trypsinized, pelleted and trypan blue positive cells were
20 enumerated. As shown in Figure 7, none of the three compounds
had any effect on the apoptosis of the wild type HeLa cells, but
40 they potently inhibited etoposide-induced apoptosis in HeLa cells
transfected with the estrogen receptor α or estrogen receptor β .

45

25

EXAMPLE 7

50

55

5 The mechanism of the anti-apoptotic effect of the
estrogenic compounds described herein was established by
demonstrating that 17 α -estradiol, 17 β -estradiol, 17 β -estradiol-
10 BSA or estratriene-3-ol, at 10⁻⁸ M concentrations, activated
5 extracellular signal regulated kinases (ERKs). In this experiment,
MLO-Y4 osteocytic cells were incubated for 25 minutes in serum-
15 free medium. Subsequently, 17 α -estradiol, 17 β -estradiol, 17 β -
estradiol-BSA or estratriene-3-ol (10⁻⁸ M) were added and cells
incubated for an additional 5, 15, or 30 minutes. Cell lysates were
20 prepared and proteins were separated by electrophoresis in
polyacrylamide gels and transferred to PVDF membranes.
Western blotting was performed using a specific antibody
25 recognizing phosphorylated extracellular signal regulated kinases
1 and 2, followed by reblotting with an antibody recognizing total
15 extracellular signal regulated kinases. Blots were developed by
enhanced chemiluminescence. As shown in Figure 8, all these
30 compounds specifically increased the phosphorylated fraction of
ERK1/2 without affecting the total amount of ERK1/2. This effect
is too rapid to be accounted for by the classical mechanism of
35 estrogen action. Instead, it is consistent with a non-genomic
20 action mediated via membrane-associated estrogen receptors, as
suggested by the experiments presented in Examples 4, 5 and 6.

EXAMPLE 8

25 The ability of 17 α -estradiol, 17 β -estradiol, 17 β -
45 estradiol-BSA or estratriene-3-ol to activate ERKs was abrogated
in the presence of the specific inhibitor of ERK kinase, PD98059.

5 In this experiment, MLO-Y4 osteocytic cells were incubated for 25
minutes in serum-free medium in the presence or absence of 50
10 μ M PD98059. Subsequently, 17α -estradiol, 17β -estradiol, 17β -
estradiol-BSA or estratriene-3-ol (10^{-8} M) were added and cells
5 incubated for another 5 minutes. Cell lysates were prepared and
proteins were separated by electrophoresis in polyacrylamide gels
15 and transferred to PVDF membranes. Western blotting was
performed using a specific antibody recognizing phosphorylated
extracellular signal regulated kinases 1 and 2, followed by
20 10 reblotting with an antibody recognizing total extracellular signal
regulated kinases. Blots were developed by enhanced
chemiluminescence.

25 EXAMPLE 9

15
30 That indeed the anti-apoptotic effect of all the
compounds tested herein was mediated via activation of ERKs was
established by the results of the experiments shown in Figure 10.
35 In this experiment, MLO-Y4 osteocytic cells were pretreated for 1
20 hour with the specific inhibitor of ERKs activation, PD98059,
before the addition of 10^{-8} M 17α -estradiol, 17β -estradiol, or 17β -
40 estradiol-BSA. Apoptosis was induced by incubation with the pro-
apoptotic agent dexamethasone for 6 hours and quantified as
described in Figure 3. PD98059 prevented the anti-apoptotic
25 effect of all three compounds tested in this experiment.

45 In conclusion, the results of the examples provided
above demonstrate that loss of estrogen *in vivo* leads to several-
fold increase in the prevalence of apoptosis of osteoblasts and
50

5 osteocytes. Consistent with the *in vivo* findings, 17 α -estradiol, as
well as 17 β -estradiol, 17 β -estradiol-BSA and estratriene-3-ol
10 inhibit the apoptosis of osteoblastic cells derived from murine
calvaria or osteocytes, represented herein by the cell line MLO-Y4.
5 The anti-apoptotic effect of all these compounds requires the
presence of either estrogen receptor α or estrogen receptor β and
15 is mediated via the ability of these compounds to activate specific
MAP kinases, namely the extracellular signal regulated kinases
(ERKs).

10

EXAMPLE 10

25 Similar to the results with estrogenic compounds,
androgenic compounds also inhibited apoptosis of osteoblastic cells
15 derived from murine calvaria induced by etoposide (Table 3). In
these experiments, cells were pretreated with the indicated
30 concentrations of the various compounds for 1 hour, in the
absence or presence of the androgen receptor antagonist
flutamide, before the addition of the proapoptotic agent etoposide.
35 20 Apoptosis was determined after 6 hours by trypan blue uptake as
described in Figure 3. Notably, as in the case of estrogenic
40 compounds, all these effects were apparently mediated by the
androgen receptor, as evidenced by the inhibition of the anti-
apoptotic effects of the androgenic compounds by a specific
25 androgen receptor antagonist. Moreover, and as in the case of
45 estrogens, the androgen receptor-mediated protection of
etoposide-induced apoptosis was seen with a membrane
impermeable androgen (testosterone-17 β -hemisuccinate
50

conjugated with BSA), strongly suggesting the existence of a membrane-associated androgen receptor, analogous to the membrane-associated estrogen receptor.

Table 3 Inhibition of etoposide-induced osteoblast apoptosis by androgens and progestins		
Compound	Lowest Effective Concentration	Suppression by 10⁻⁸ M Flutamide
Testosterone	10 ⁻⁹ M	yes
Testosterone 17 β -Hemisuccinate: BSA	10 ⁻⁸ M	yes
5- α -dihydrotestosterone	10 ⁻⁹ M	yes
5- β -dihydrotestosterone	10 ⁻¹⁰ M	yes
Dehydroisoandrosterone-3-sulfate (DHES)	10 ⁻⁸ M	no*
4-androstene-3,17-dione	10 ⁻⁸ M	yes
5-androstene-3 β -17 α -diol	10 ⁻⁸ M	yes
RU1881	10 ⁻⁸ M	yes

* Flutamide did block the anti-apoptotic effect of DHES at higher (10⁻⁷ M) concentration.

EXAMPLE 11

5 That the anti-apoptotic effects of estrogenic
compounds is dissociated from their transcriptional activity was
established by demonstrating that even though estratriene-3-ol
10 was as potent as 17 β estradiol in inhibiting apoptosis, unlike 17 β
5 estradiol, it did not transactivate an estrogen response element
through the estrogen receptor α . In this experiment, hER α was
15 overexpressed in 293 cells (which lack constitutive ER α) along
with a reporter construct containing 3 copies of an estrogen
response element driving the luciferase gene. Light units were
20 10 counted and normalized to coexpressed β -galactosidase activity to
control for differences in transfection efficiency.

25 EXAMPLE 12

15 Herein, a general experimental protocol for studies
aiming to evaluate compounds with anti-apoptotic efficacy, but
30 decreased transcriptional activity (*e.g.*, estratriene-3-ol) on
osteoblasts and osteocytes in animal models is provided.
According to this design, estrogen-replete or estrogen-deficient
35 20 mice, rats, dogs, primates, etc., or animals representing models of
involutional osteoporosis and/or defective osteoblastogenesis (*e.g.*,
40 the senescence accelerated mouse, SAMP6: (Jilka et al., J Clin
Invest 97:1732-1740, 1996)), or animal models of glucocorticoid
excess (*e.g.*, Weinstein et al. J Clin Invest, 102:274-282, 1998) are
45 25 administered estratriene-3-ol or other test compound to
determine whether they can suppress osteoblast and osteocyte
apoptosis and whether changes in apoptosis would be associated

with changes in BMD, bone formation rate, or cancellous bone volume.

In a representative experiment of this sort, six 4-5 month old female mice per group are screened twice for BMD in a four week period immediately prior to the initiation of the experiment to establish that peak adult bone mass has been attained. A subset of mice are then ovariectomized. Intact and ovariectomized mice are treated with vehicle, or 20, 200 or 2000 ng/g body weight estratriene-3-ol or another test compound. Ovariectomized mice are also treated with 20 ng/g body weight 17 β -estradiol for comparison purposes.

Stock solutions of the test agents (10,000 μ g/ml) are maintained in approximately 2.0 ml of 95% ethanol. These stocks are diluted in 95% ethanol to make 1000 μ g/ml and 100 μ g/ml concentrations. The concentration of the stocks is checked spectrophotometrically. For each animal injection, the test agent is diluted in sesame oil and sonicated. Test agents are administered for 28 days by subcutaneous injections on alternative days. The mice are weighed weekly and serum samples are collected at appropriate times for analysis of bone biochemical markers, such as osteocalcin or collagen cross-links. Tetracycline labeling is performed by administration of the antibiotic (30 mg/kg) at 2 and 8 days prior to the end of each experiment. Table 1 shows a representative example of 25 g mice divided into 5 groups with each animal receiving 100 μ l of the test agent per injection.

TABLE 1

Treatment	Injection
-----------	-----------

	(steroid + sesame oil)
vehicle	100 μ l 95% ethanol + 1900 μ l
20 ng/g estratriene-3-ol	100 μ l 100 μ g/ml stock + 1900 μ l
200 ng/g estratriene-3-ol	100 μ l 1000 μ g/ml stock + 1900 μ l
2000 ng/g estratriene-3-ol	100 μ l 10,000 μ g/ml stock + 1900 μ l
20 ng/g 17 β -estradiol	50 μ l 100 μ g/ml stock + 950 μ l

During the 28 day experiment, BMD is determined in live animals at day 0, 14 and 28. Following animal sacrifice at the end of the experiment, the vertebral bones L1-L4 are collected for fixation and embedded undecalcified in methylmethacrylate plastic for the determination of the prevalence of osteoblast and osteocyte apoptosis and other static and dynamic histomorphometric measurements. L5 vertebrae are isolated for determining anti-fracture efficacy of the compounds by assaying compression, 3 point bending and other appropriate biomechanical tests. Results confirming the expected efficacy of these compounds show decreased prevalence of osteoblast and/or osteocyte apoptosis, and/or positive BMD changes, and/or increased cancellous bone area, and/or increased rate of bone formation, and/or increased biomechanical strength.

As an example, the results of an experiment whereby 2000 ng/g body weight of estratriene-3-ol was administered for 28 days to estrogen-replete (intact) or estrogen-deficient (ovariectomized) mice are shown in Table 2.

Increased BMD by estratriene-3-ol administration
intact-vehicle;

5 intact-2000 ng/g 3-ol:

vehicle vs 2000 ng/g:

10 **Ovx-vehicle:**

global	1hindquart	spine 1	global	2 hindquart	spine 2
0.0525	0.0539	0.0550	0.0493	0.0507	0.0548
0.0479	0.0484	0.0538	0.0497	0.0569	0.0545
0.0510	0.0543	0.0565	0.0483	0.0509	0.0545
0.0538	0.0548	0.0583	0.0483	0.0515	0.0536

5	0.0567	0.0632	0.0620	0.0538	0.0584	0.0588	
	0.0533	0.0533	0.0572	0.0504	0.0507	0.0559	
	0.0543	0.0592	0.0583	0.0491	0.0526	0.0545	
	0.0490	0.0518	0.0551	0.0475	0.0487	0.0534	
10	0.0523	0.0550	0.0570	0.0496	0.0528	0.0550	(mean)
	0.0029	0.0049	0.0026	0.0019	0.0035	0.0017	(std)
	0.0567	0.0632	0.0620	0.0538	0.0584	0.0588	(max)

Ovx-2000 ng/g 3-ol:

15	global	1hindquart	spine 1	global	2 hindquart	spine 2	
	0.0505	0.0527	0.0547	0.0565	0.0602	0.0608	
	0.0542	0.0588	0.0581	0.0557	0.0632	0.0585	
	0.0496	0.0504	0.0542	0.0548	0.0599	0.0584	
20	0.0540	0.0596	0.0586	0.0545	0.0624	0.0598	
	0.0526	0.0547	0.0580	0.0564	0.0598	0.0610	
	0.0569	0.0604	0.0628	0.0568	0.0647	0.0625	
	0.0565	0.0591	0.0603	0.0550	0.0630	0.0578	
	0.0528	0.0582	0.0568	0.0539	0.0599	0.0605	
25	0.0534	0.0573	0.0579	0.0555	0.0618	0.0599	(mean)
	0.0026	0.0036	0.0028	0.0011	0.0020	0.0016	(std)
	0.0569	0.0604	0.0628	0.0568	0.0647	0.0625	(max)
	0.0496	0.0504	0.0542	0.0539	0.0598	0.0578	(min)

ovx vs 2000 ng/g:

30	t-test	0.0000	0.0001	0.0001
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5

Each row represents values for individual animals.

The first three sets of numbers represent the initial BMD measurements (by dual-energy x-ray absorptiometry with Hologic QDR2000 plus, using customized software) at day 0 and the last three BMD measurements at the end of the experiment. Global = BMD of the entire skeleton minus the head and tail; hindquarters = the mean BMD of both hindlimbs; spine = the BMD of cervical, thoracic and lumbar spine.

10

EXAMPLE 13

Herein, a general experimental protocol evaluating the anti-fracture efficacy of compounds like estratriene-3-ol is provided. According to this design, estrogen-replete or estrogen-deficient mice, rats, dogs, primates, etc., or animals representing models of involutional osteoporosis and/or defective osteoblastogenesis (*e.g.*, the senescence accelerated mouse, SAMP6: (Jilka et al., J Clin Invest 97:1732-1740, 1996)), or animal models of glucocorticoid excess (*e.g.*, Weinstein et al. J Clin Invest, 102:274-282, 1998) are administered estratriene-3-ol to determine whether they can increase bone strength.

In a representative experiment of this sort, seven 4-5 month old female mice per group are screened twice for BMD in a four week period immediately prior to the initiation of the experiment to establish that peak adult bone mass has been attained. A subset of mice are then ovariectomized. Intact and ovariectomized mice are treated with vehicle, or 20, 200 or 2000 ng/g body weight estratriene-3-ol or another ANGEL compound. Ovariectomized mice are also treated with 20 ng/g body weight 17 β -estradiol for comparison purposes. Ultimate load bearing properties of the fifth lumbar murine vertebrae (L5) is determined. This is done using a servohydraulic axial-torsional material testing machine (Model MTS 810 Bionx; MTS Systems Corp., Eden Prairie, MN) and a Lebow load cell (Eaton Products, Troy, MI). Data are recorded and analyzed using the LabVIEW software package and an acquisition/signal conditioning board (Model NB-MIO-16, National Instruments Corporation, Austin, TX).

The L5 specimens that is used for ultimate load bearing is cleaned of surrounding soft tissue and the length and diameter recorded with a digital caliper at a resolution of 0.01 mm (Mitutoyo Model #500-196, Ace Tools, Ft. Smith, AR). The vertebrae are wrapped in saline-soaked gauze throughout preparation and testing and stored overnight at 4°C before testing. Vertebrae are individually compressed between parallel loading platens along the cephalocaudal axis until failure and the ultimate load (in Newtons) and displacement (in mm) are recorded.

As an example, the results of an experiment whereby 2000 ng/g body weight of estradiene-3-ol was administered for 28 days to estrogen-replete (intact) or estrogen-deficient (ovariectomized) mice (from the same animals shown in Example 12) is shown in Table 4.

Table 4 Changes in Vertebral Compression Strength (VCS*), Induced by <i>In vivo</i> Administration of E-3-ol: Demonstration of Greater Increase in CVS than BMD (n = 7 per group)		
	Vertebral Compression (Newtons)	Global BMD (g/cm²)
Intact- vehicle	66.78 ± 17.47 50.3 ± 7.58	0.0508 ± 0.0026
Ovx- vehicle	96.26 ± 15.92 (<i>p</i> <0.006) 85.57 ± 10.17	0.0486 ± 0.0011 0.0554 ± 0.0015 (<i>p</i> <0.002)
Intact-E- 3-ol	(<i>p</i> <0.00001)	0.0555 ± 0.0011

Ovx-E-3- ol		($p < 0.00001$)
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*Each value represents the mean from seven animals.

The BMD values shown for comparison here are from the experiment described in Example 12.

EXAMPLE 14

To determine whether the anti-apoptotic effects of estrogenic compounds are mechanistically dissociable from their transcriptional effects, specific conformational changes of the receptor protein leading to prevention of apoptosis versus transcriptional activity were sought. The rationale behind these studies was based on recent evidence that the transcriptional activity of the ER is greatly dependent on ligand-induced conformational changes of the receptor protein. Indeed, using phage display libraries, McDonnell and co-workers have recently screened for and isolated four classes of small (11 amino acids) peptides that recognize distinct conformational changes of the estrogen receptor, and can either selectively block transcription from specific ligands (e.g., estradiol but not tamoxifen and vice versa) or selectively block ER α but not ER β -mediated transcription, and vice versa, when tested on a consensus ERE

5 (Norris et al. Science 285:744-746, 1999). The first class contains
the LXXLL motif and can interact with both estradiol-activated
ER α and ER β . The second class displays specific interaction with
10 estradiol- and tamoxifen-activated ER α , whereas the third class
5 can interact specifically with tamoxifen-activated ER β . Yet a fourth
class with a SREWFXXLL conserved motif was found to complex to
15 tamoxifen-activated ER α and ER β . Indeed, when fusion proteins
made with these peptides and the Gal4-DNA binding domain and
were co-expressed with ER in HeLa cells they functioned as
20 10 ligand-receptor complex-specific antagonists, demonstrating that
ligand activation triggers transcriptional activity by conferring
specific conformational changes on the receptor protein (Paige LA,
25 Christensen DJ, Gron H, Norris JD, Gottlin EB, Padilla KM, Change C-
Y, Ballas LM, Hamilton PT, McDonnell DP, Fowlkes DM. Estrogen
15 receptor (ER) modulators each induce distinct conformational
changes in ER α and ER β . *Proc. Natl. Acad. Sci* 96:3999-4004, 1999).

Based on the findings that estrogenic compounds like
the conjugated 17- β estradiol with BSA have, at least as potent
35 anti-apoptotic effects as estrogen while have significantly
20 decreased transcriptional activity, the hypothesis that the non-
genomic anti-apoptotic effects of estrogen can be initiated by
40 distinct ligand-dependent conformational changes of the ER, as
compared to the conformational changes required for the
transcriptional effects of the ER was tested. It was found that
45 25 indeed there is dissociation of conformational changes. Based on
this, one can explain the mechanistic basis of the apparent
dissociation of the two sets of actions. This knowledge forms the
50 basis for the design of the screening strategies described herein for

5 ligands which display non-transcriptional effects, but lack the
ability to initiate transcriptional activation.

10 One skilled in the art will readily appreciate that the
present invention is well adapted to carry out the objects and
5 obtain the ends and advantages mentioned, as well as those
objects, ends and advantages inherent herein. The present
15 examples, along with the methods, procedures, treatments,
molecules, and specific compounds described herein are presently
representative of preferred embodiments, are exemplary, and are
20 10 not intended as limitations on the scope of the invention. Changes
therein and other uses will occur to those skilled in the art which
are encompassed within the spirit of the invention as defined by
25 the scope of the claims.

Claims

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WHAT IS CLAIMED IS:

1. A method for increasing bone mass at least 10% in a host without a loss in bone strength or quality is provided that includes administering an effective amount of a compound that (i) binds to the estrogen α or β receptor (or the equivalent receptor in the host animal) with an association constant of at least 10^8 M^{-1} , and preferably, at least 10^{10} M^{-1} ; (ii) (a) induces estrogenic gene transcriptional activity at a level that is no greater than 10% that of 17 β -estradiol, and preferably no greater than 5, 1 or even 0.1% that of 17 β -estradiol when administered *in vivo* at concentrations of 10^{-11} to 10^{-7} M a dosage of at least 0.1 ng/kg body weight or *in vitro* in osteoblastic or osteocytic cells with natural estrogen receptors or cells transfected with estrogen receptors or (b) induces an increase in uterine weight of no more than 10% that of 17 β -estradiol (or the equivalent compound in a host animal); (iii) induces the phosphorylation of extracellular signal regulated kinase (ERK) when administered *in vivo* at a dosage of at least 0.1 ng/kg body weight or *in vitro* at concentrations of 10^{-11} to 10^{-7} M in osteoblastic cells with natural estrogen receptors or cells transfected with estrogen receptors; and (iv) has an anti-apoptotic effect on osteoblasts at an *in vivo* dosage of at least 0.1 ng/kg body weight *in vitro* in osteoblastic or osteocytic cells with natural estrogen receptors or cells transfected with estrogen receptors.

2. The method of claim 1, wherein the compound is not an estrogen compound.

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3. The method of claim 1, wherein the compound is an estrogen.

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5 4. The method of claim 3, wherein the estrogen compound is converted to a nonestrogen by attaching a substituent which prevents the compound from entering the cell but does not significantly affect the binding of the compound to the estrogen cell-surface receptor.

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5. A method for increasing bone mass at least 10% in a host without a loss in bone strength or quality is provided that includes administering an effective amount of a compound that (i) binds to the androgen receptor (or the equivalent receptor in the host animal) with an association constant of at least 10^8 M^{-1} , and preferably, at least 10^{10} M^{-1} ; (ii) (a) induces androgenic gene transcriptional activity at a level that is no greater than 10% that of testosterone, and preferably no greater than 5, 1 or even 0.1% that of testosterone when administered *in vivo* at a dosage of at least 0.1 ng/kg body weight or *in vitro* at concentrations of 10^{-11} to 10^{-7} M in osteoblastic cells with the natural androgen receptor or cells transfected with the androgen receptor or (b) induces an increase in muscle weight or virilization in women of no more than 10% that which is induced by testosterone (or the equivalent compound in a host animal); (iii) induces the phosphorylation of extracellular signal regulated kinase (ERK) when administered *in vivo* at a dosage of at least 0.1 ng/kg body weight or *in vitro* in osteoblastic cells with the natural androgen receptor or cells

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5 transfected with the androgen receptor; and (iv) has an anti-
apoptotic effect on osteoblasts at an *in vivo* dosage of at least 0.1
ng/kg body weight or *in vitro* in osteoblastic cells with the natural
10 androgen receptor or transfected with the androgen receptor.

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6. The method of claim 6, wherein the compound is
15 not an androgen.

7. The method of claim 6, wherein the compound is
20 10 an androgen.

8. The method of claim 8, wherein the androgen is
25 converted to a nonandrogen by attaching a substituent which
prevents the compound from entering the cell but which does not
15 significantly affect the ability of the compound to bind to the
androgen cell-surface receptor.
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9. The method of claim 1, wherein the compound
35 also has a pro-apoptotic effect on osteoclasts at an *in vivo* dosage
20 of at least 0.1 ng/kg body weight, or in osteoclastic cells with
natural estrogen receptors or cells transfected with estrogen
40 receptors.

10. The method of claim 7, wherein the compound
25 also has a pro-apoptotic effect on osteoclasts at an *in vivo* dosage
45 of at least 0.1 ng/kg body weight, or in osteoclastic cells with
natural estrogen receptors or cells transfected with estrogen
50 receptors.

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11. A method for selecting a compound that increases bone mass in a host at least 10% without a loss in bone strength or quality is provided that includes evaluating whether the compound (i) binds to the estrogen or androgen receptor (or the equivalent receptor in the host animal) with an association constant of at least 10^8 M^{-1} , and preferably, at least 10^{10} M^{-1} ; (ii) (a) induces estrogenic or androgenic gene transcriptional activity at a level that is no greater than 10% that of 17β -estradiol or testosterone, and preferably no greater than 5, 1 or even 0.1% that of 17β -estradiol or testosterone, as appropriate, when administered *in vivo* at a dosage of at least 0.1 ng/kg body weight or *in vitro* in osteoblastic cells with the natural androgen or estrogen receptor or cells transfected with the androgen or estrogen receptor or (b) induces an increase in uterine weight of no more than 10% that which is induced by 17β -estradiol or muscle weight or virilization in women of no more than 10% that which is induced by testosterone (or the equivalent compound in a host animal); (iii) induces the phosphorylation of extracellular signal regulated kinase (ERK) when administered *in vivo* at a dosage of at least 0.1 ng/kg body weight or *in vitro* in osteoblastic or osteocytic cells with the natural androgen or estrogen receptor or cells transfected with the androgen or estrogen receptor; and (iv) has an anti-apoptotic effect on osteoblasts at an *in vivo* dosage of at least 0.1 ng/kg body weight or *in vitro* in osteoblastic cells with the natural androgen or estrogen receptor or cells transfected with the androgen or estrogen receptor.

5 12. A method for screening for compounds that
possess bone anabolic effects, comprising the steps of: a)
contacting a sample of osteoblast cells with a compound; and b)
10 comparing the number of osteoblast cells undergoing apoptosis in
5 the compound-treated cells with the number of osteoblast cells
undergoing apoptosis in an untreated sample of osteoblast cells.

15 13. A method for conferring bone protection on a
population of cells in a subject through osteoblast/osteocyte anti-
20 apoptotic effects, comprising the step of: administering an
effective dose of a compound to said population of cells, wherein
said compound has a terminal phenol group and at least a second
25 ring, wherein said compound has a molecular weight of less than
1000.

15 14. The method of claim 14, wherein said compound
30 has a molecular weight greater than 170.

35 15. The method of claim 14, wherein said terminal
20 phenyl ring is a non-steroidal compound.

40 16. The method of claim 16, wherein said terminal
phenyl ring is a phenolic A ring.

25 17. The method of claim 14, wherein said effective
45 dose of said compound results in a plasma concentration of less
than 500 nM.

5 18. The method of claim 18, wherein said plasma concentration is from about 0.02 nM to about 500 nM.

10 19. The method of claim 19, wherein said plasma
5 concentration is from about 0.1 nM to about 1 nM.

15 20. The method of claim 14, wherein said compound is selected from the group consisting of a four-ring structure, a three-ring structure and a two-ring structure.

20 21. The method of claim 21, wherein when said
10 compound is a four-ring structure, said effective dose is that which achieves a plasma concentration of less than 500 nM.

15 22. The method of claim 21, wherein when said
30 compound is a three-ring structure, said three-ring structure is a phenanthrene compound.

35 23. The method of claim 23, wherein said
20 phenanthrene compound is selected from the group consisting of a tetrahydrophenanthrene and an octahydrophenanthrene.

40 24. The method of claim 23, wherein said
phenanthrene compound is selected from the group consisting of a
25 phenanthrenemethanol and a phenanthrenecarboxyaldehyde.

45 25. The method of claim 21, wherein when said
50 compound is a two-ring structure, said two-ring structure is fused.

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26. The method of claim 26, wherein said fused two-ring structure is selected from the group consisting of naphthol and naphthalene.

27. The method of claim 21, wherein when said compound is a two-ring structure, said two-ring structure is non-fused.

28. The method of claim 28, wherein said non-fused two-ring structure comprises a linkage group.

29. The method of claim 14, wherein said compound is administered in combination with a reducing agent.

30. The method of claim 1, further comprising administering the compound in combination with a second pharmaceutical agent.

31. The method of claim 31, wherein the second pharmaceutical agent is bone anti-resorption agent.

32. The method of claim 31, wherein the second pharmaceutical agent is a bone mass anabolizing agent.

33. The method of claim 31 wherein the second pharmaceutical agent is an antioxidant.

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34. The method of claim 31, wherein the second pharmaceutical agent is a dietary supplement.

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35. The method of claim 31, wherein the second pharmaceutical agent increases the beneficial effect of the active compound on bone structure, strength, or mass.

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36. The method of claim 31, wherein the second pharmaceutical agent is selected from the group consisting of an anabolic steroid, a bisphosphonate, a calcitonin, an estrogen or progestogen, an anti-estrogens such as raloxifene or tamoxifene, parathyroid hormone, fluoride, Vitamin D or a derivative thereof, or a calcium preparation.

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37. The method of claim 31, wherein the second pharmaceutical agent is selected from the group consisting of alendronic acid, disodium clondronate, disodium etidronate, disodium medronate, disodium oxidronate, disodium pamidronate, neridronic acid, risedronic acid, teriparatide acetate, tiludronic acid, ipriflavone, potassium bicarbonate, progestogen, a thiazide, gallium nitrate, NSAIDS, plicamycin, aluminum hydroxide, calcium acetate, calcium carbonate, calcium, magnesium carbonate, and sucralfate.

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38. The method of claim 6, further comprising administering the compound in combination with a second pharmaceutical agent.

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5 39. The method of claim 39, wherein the second
pharmaceutical agent is bone anti-resorption agent.

10 40. The method of claim 39, wherein the second
5 pharmaceutical agent is a bone mass anabolizing agent.

15 41. The method of claim 39, wherein the second
pharmaceutical agent is an antioxidant.

20 42. The method of claim 39, wherein the second
pharmaceutical agent is a dietary supplement.

25 43. The method of claim 39, wherein the second
pharmaceutical agent increases the beneficial effect of the active
15 compound on bone structure, strength, or mass.

30 44. The method of claim 39, wherein the second
pharmaceutical agent is selected from the group consisting of an
anabolic steroid, a bisphosphonate, a calcitonin, an estrogen or
35 progestogen, an anti-estrogens such as raloxifene or tamoxifene,
20 parathyroid hormone, fluoride, Vitamin D or a derivative thereof,
or a calcium preparation.

40 45. The method of claim 36, wherein the second
25 pharmaceutical agent is selected from the group consisting of
alendronic acid, disodium clondronate, disodium etidronate,
45 disodium medronate, disodium oxidronate, disodium pamidronate,
neridronic acid, risedronic acid, teriparatide acetate, tiludronic
50

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acid, ipriflavone, potassium bicarbonate, progestogen, a thiazide,
gallium nitrate, NSAIDS, plicamycin, aluminum hydroxide, calcium
acetate, calcium carbonate, calcium, magnesium carbonate, and
sucralfate.

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FIG. 1

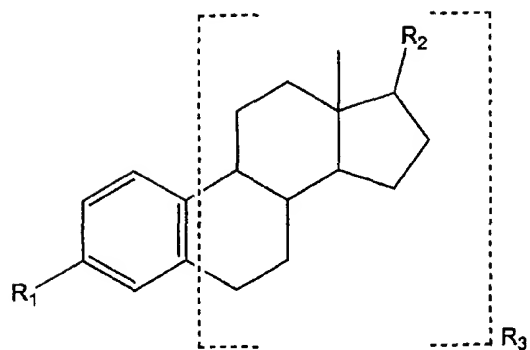
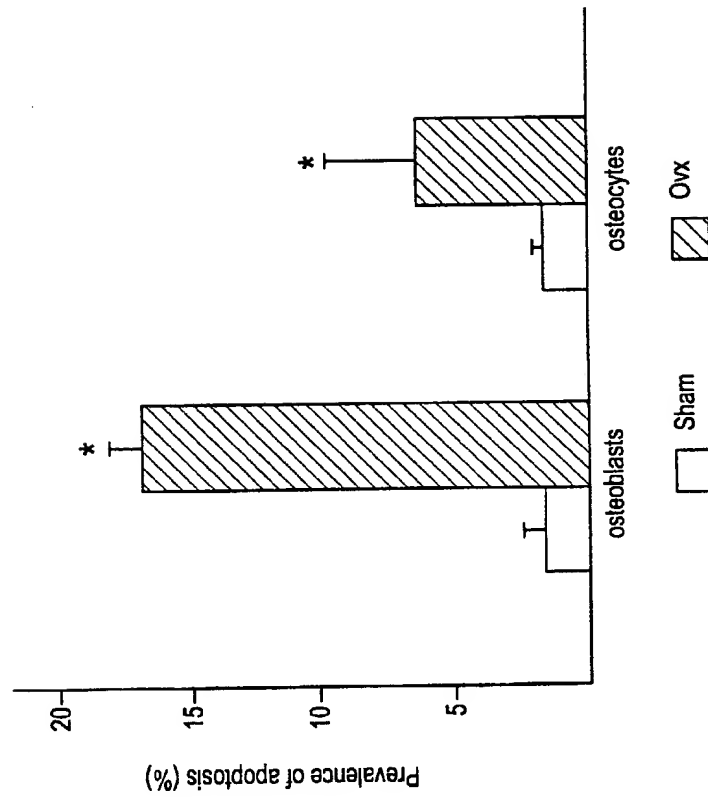
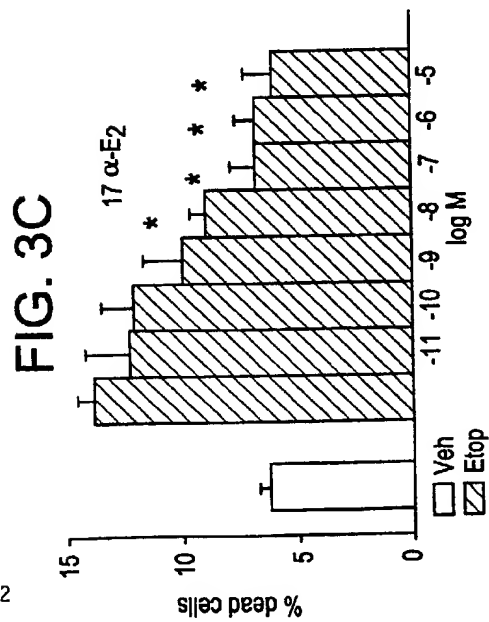
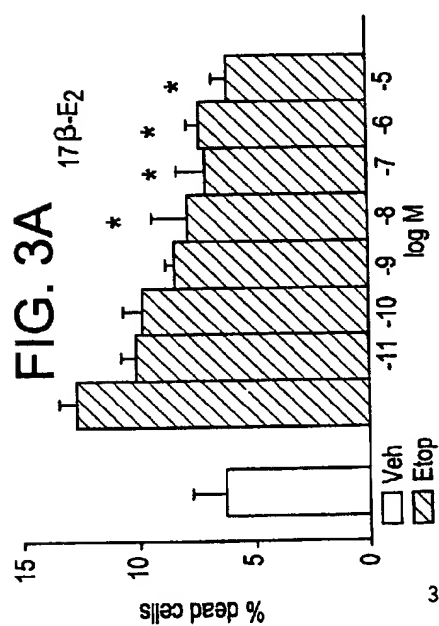
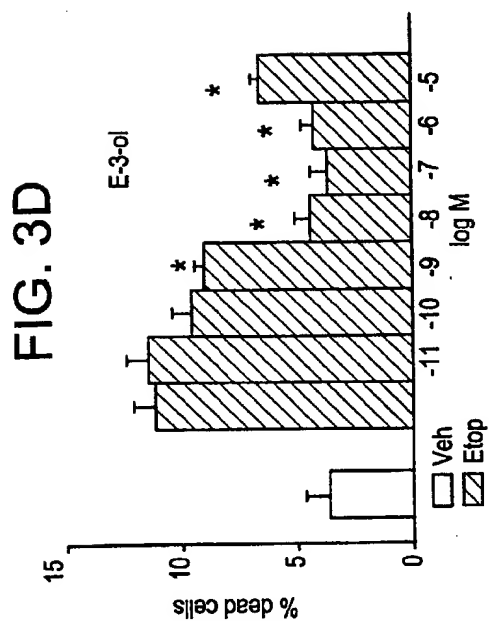
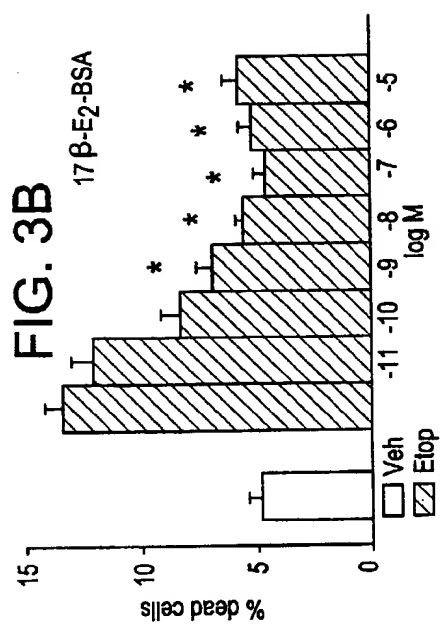


FIG. 2





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FIG. 4A

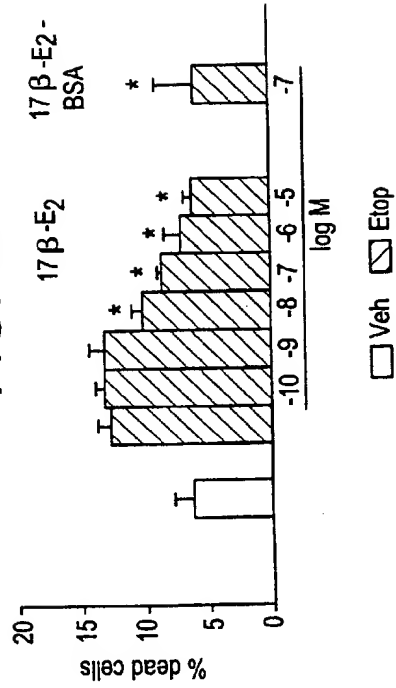


FIG. 4C

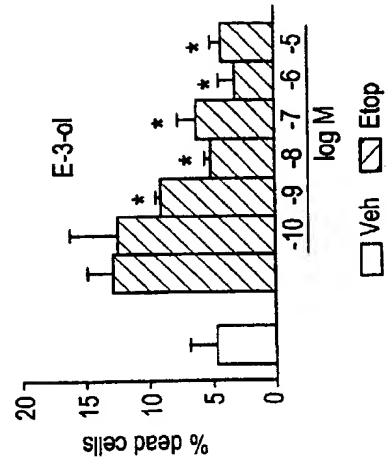
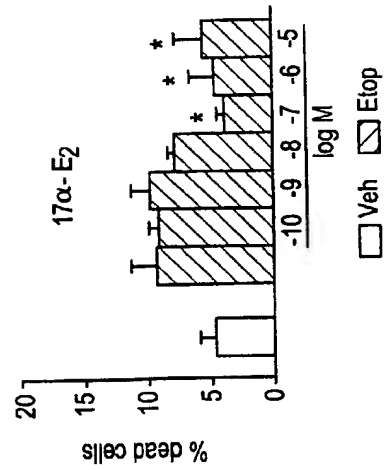
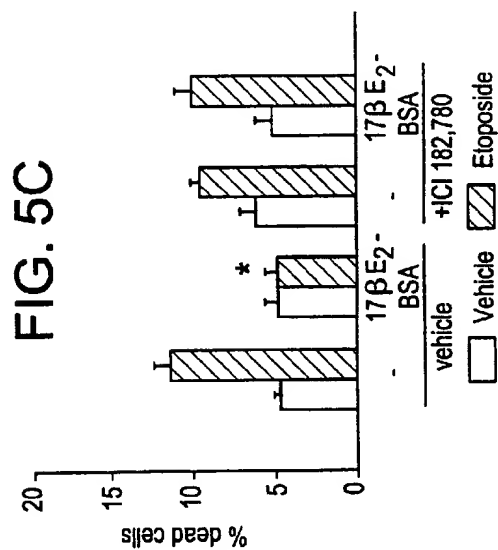
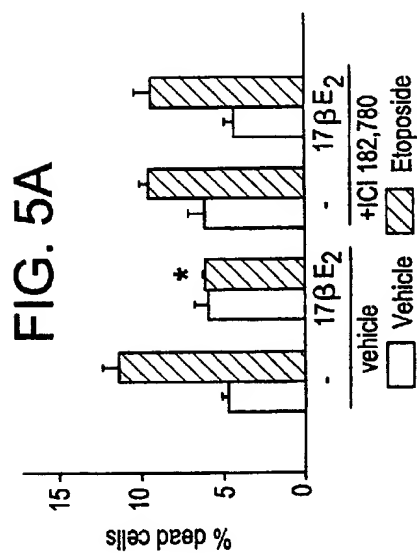
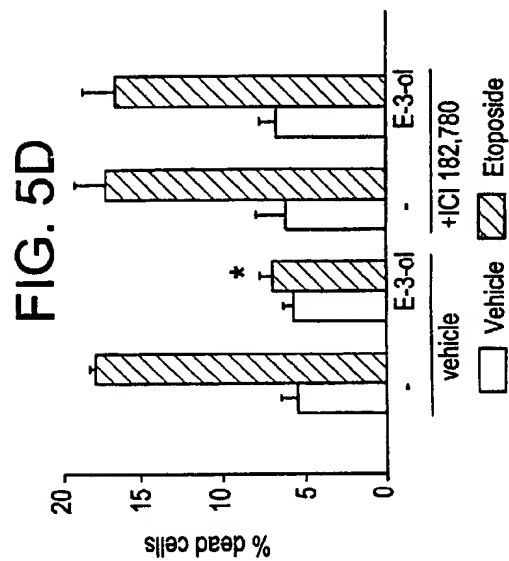
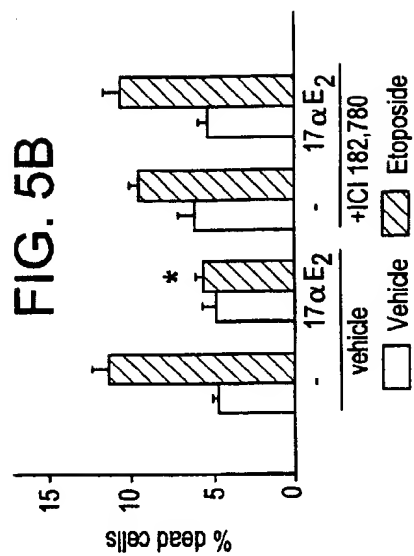


FIG. 4B





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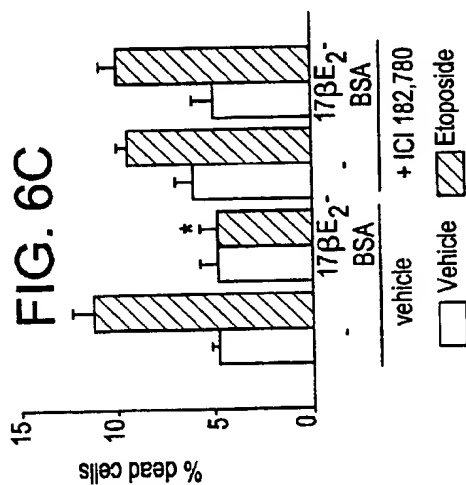
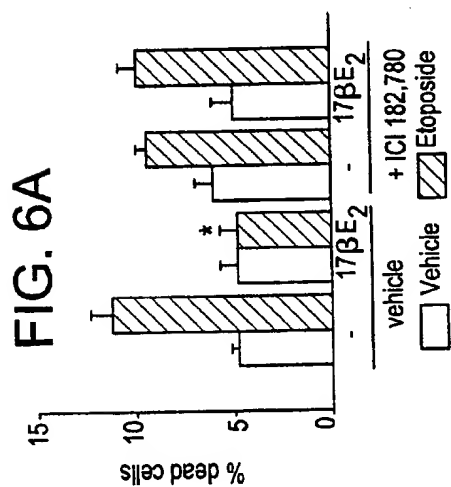
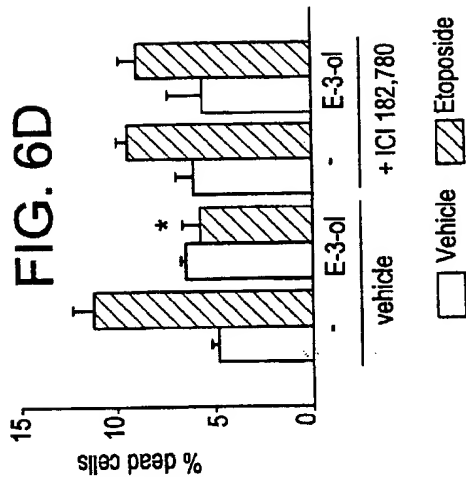
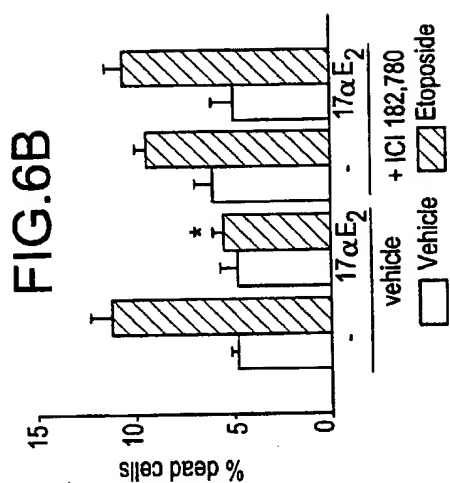
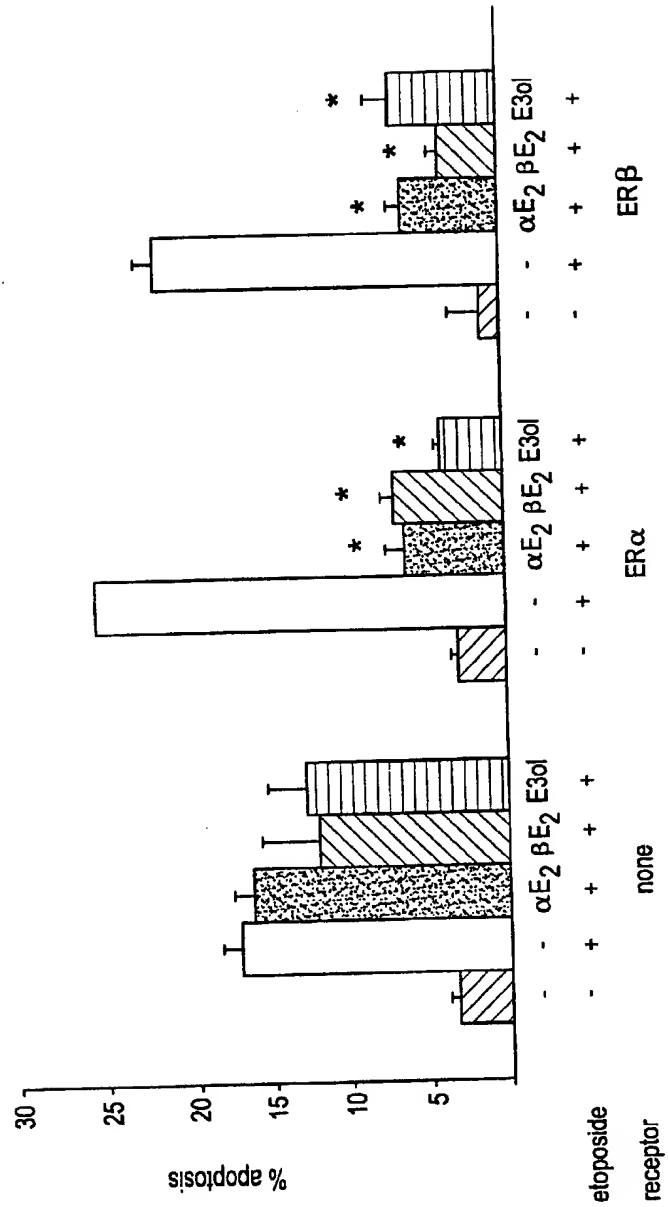
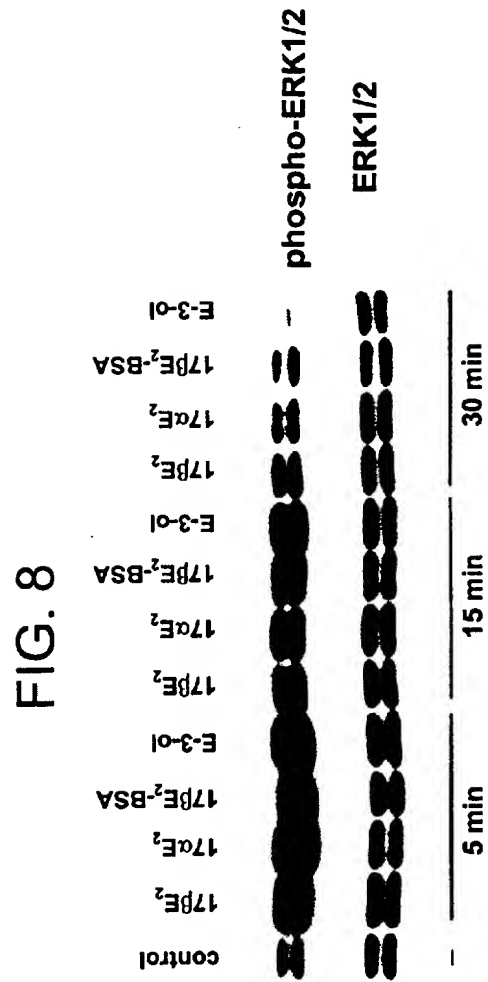
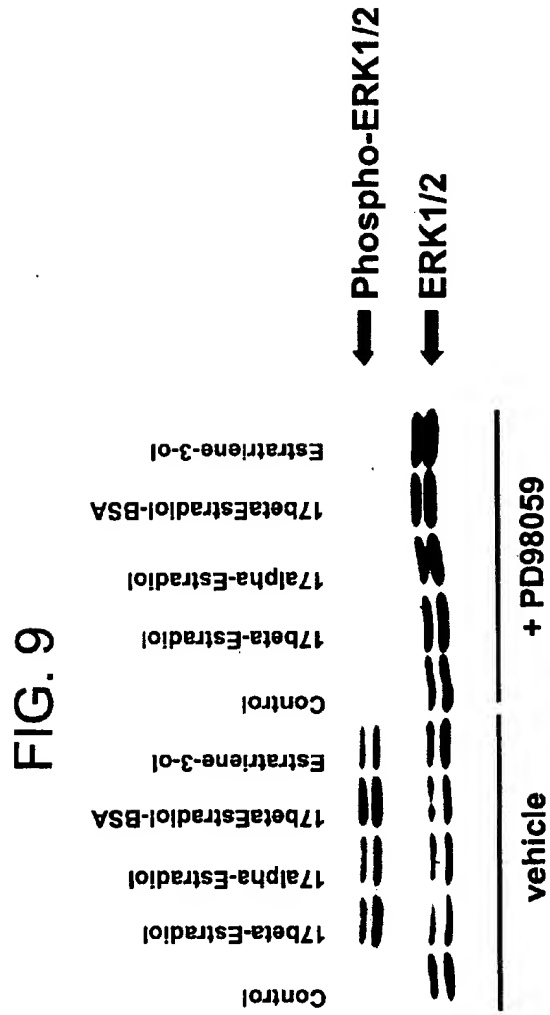
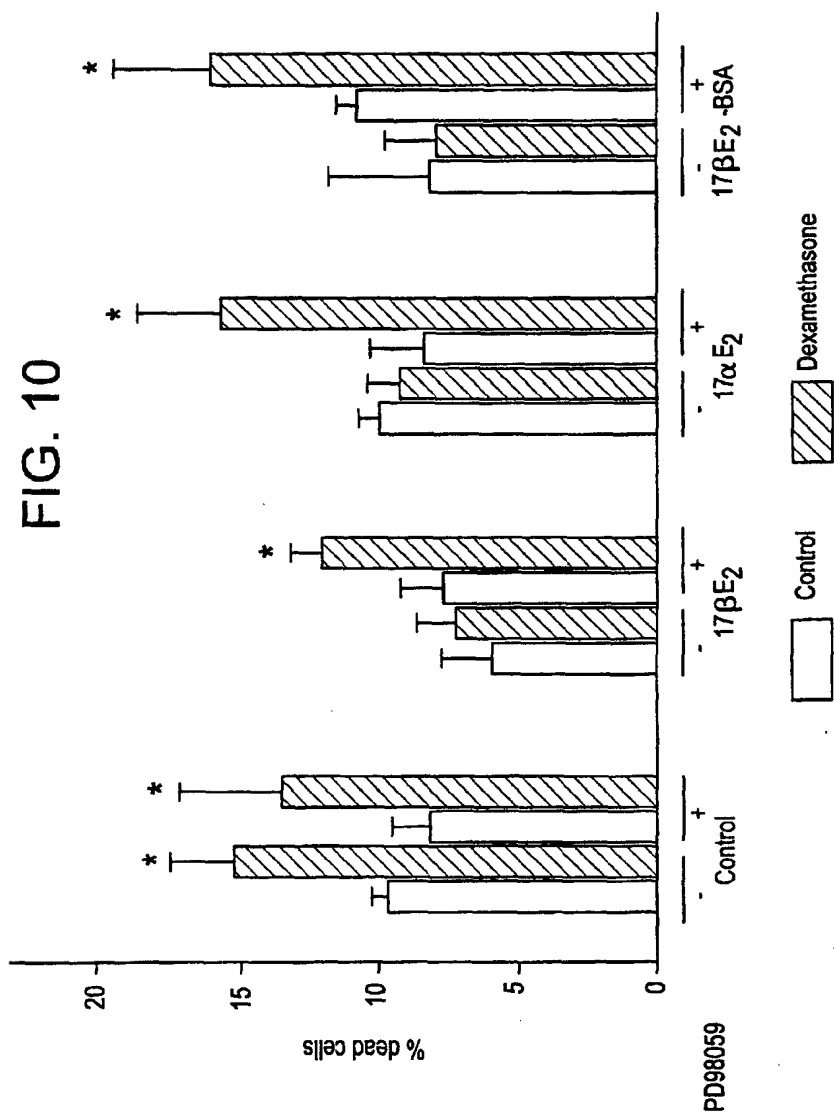


FIG. 7



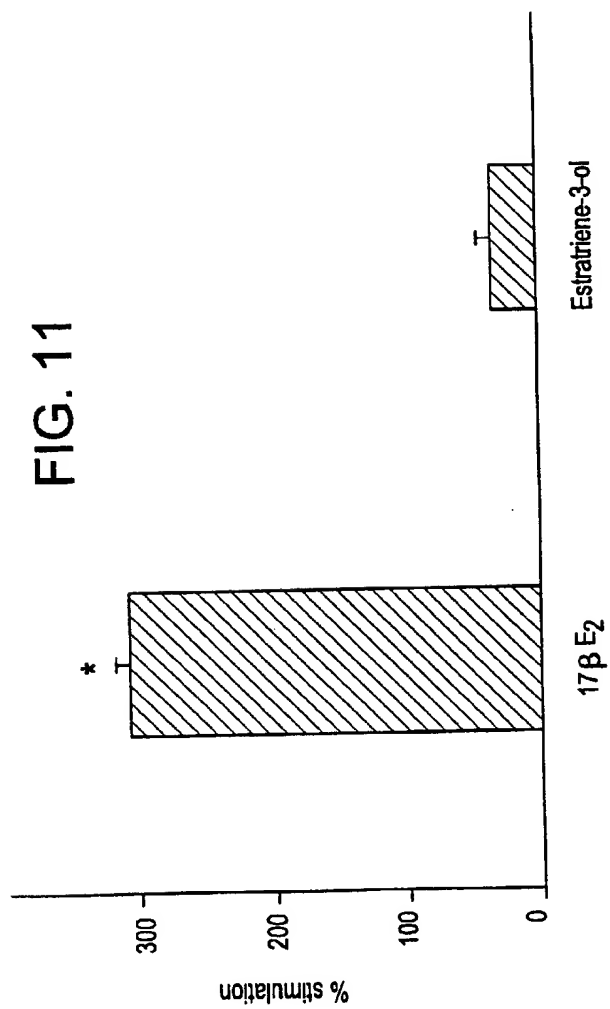






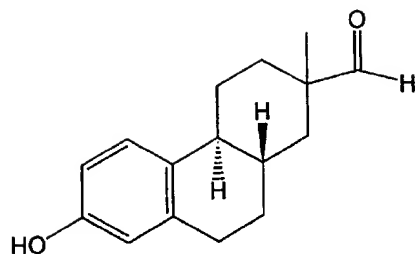
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FIG. 11

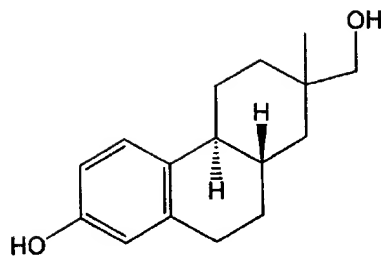


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FIG. 12



$C_{16}H_{20}O_2$
MW=244
[2S-(2a, 4a α , 10a β)]-1, 2, 3, 4, 4a, 9, 10,
10a-Octahydro-7-hydroxy-2-methyl-2-
phenanthrenecarboxaldehyde



$C_{16}H_{22}O_2$
MW=246
[2S-(2a, 4a α , 10a β)]-1, 2, 3, 4, 4a, 9, 10,
10a-Octahydro-7-hydroxy-2-methyl-2-
phenanthrenemethanol

FIG. 13

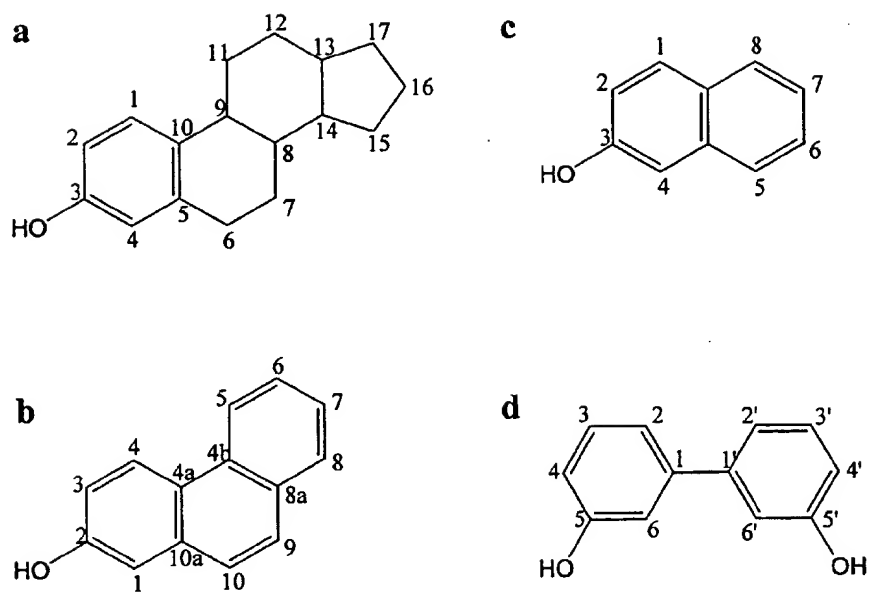


FIG. 14

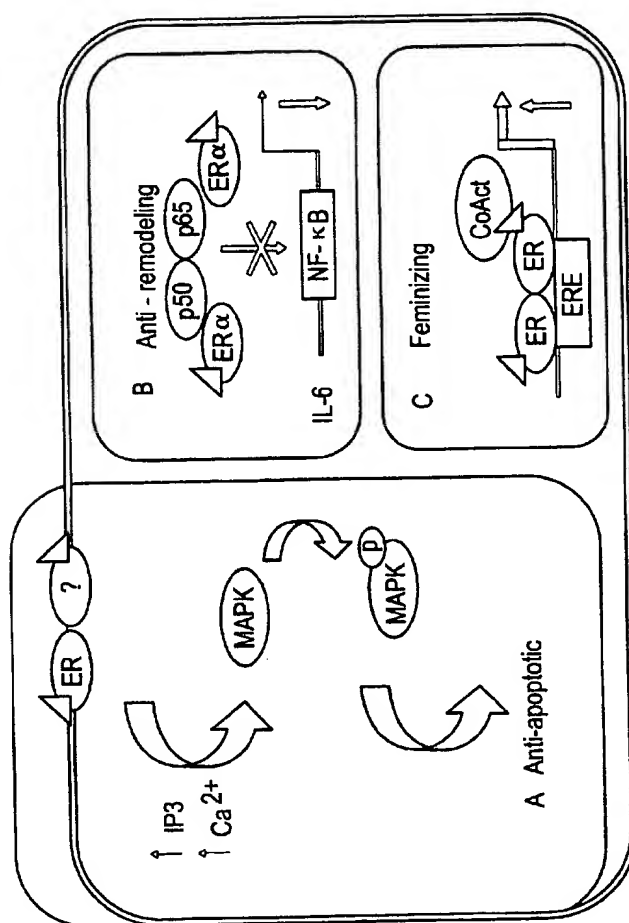


FIG. 15

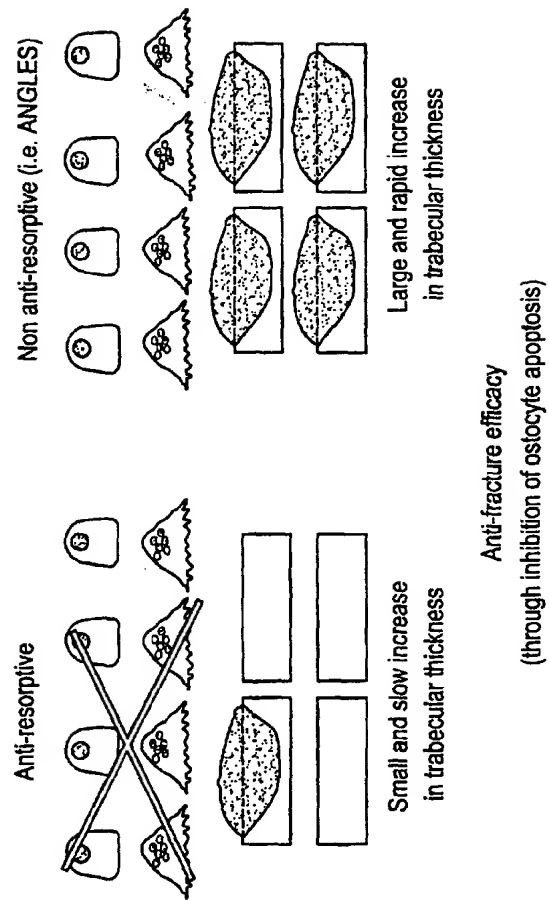
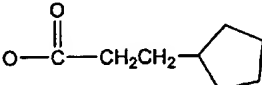


FIG. 16A

R ₁ AND/OR R ₂ SUBSTITUTIONS	
NAME	STRUCTURE
HYDROXYL	-OH
METHYL	-CH ₃
METHYL ESTER	-OCH ₃
ACETATE	
ETHYL ETHER	O-CH ₂ -CH ₃
3, 3, (OR 17, 17) DIMETHYL KETAL	
ETHYNYL- α	
BENZOATE	
BENZYL ETHER	OCH ₂ -
GLUCURONIDE	C ₆ H ₈ O ₆
SULFATE, SODIUM SALT	OSO ₃ Na
OXIDE	=O
VALERATE	-C ₅ H ₈ O
CYCLOPENTYLPROPIONATE	
PROPIONATE	
HEMISUCCINATE	-C ₄ H ₄ O ₃
PALMITATE	-C ₁₆ H ₃₂ O ₂

FIG. 16B

R ₁ AND/OR R ₂ SUBSTITUTIONS	
NAME	STRUCTURE
SODIUM PHOSPHATE	-O-PO ₃ Na ₂
ENANTHATE	-C ₇ H ₁₂ O
GLUCURONIDE, SODIUM SALT	-C ₆ H ₈ O ₆ Na
STEARATE	-C ₁₈ H ₃₄ O
TRIETHYL AMMONIUM SALT	-N-(C ₂ H ₅) ₃
CYPIONATE	

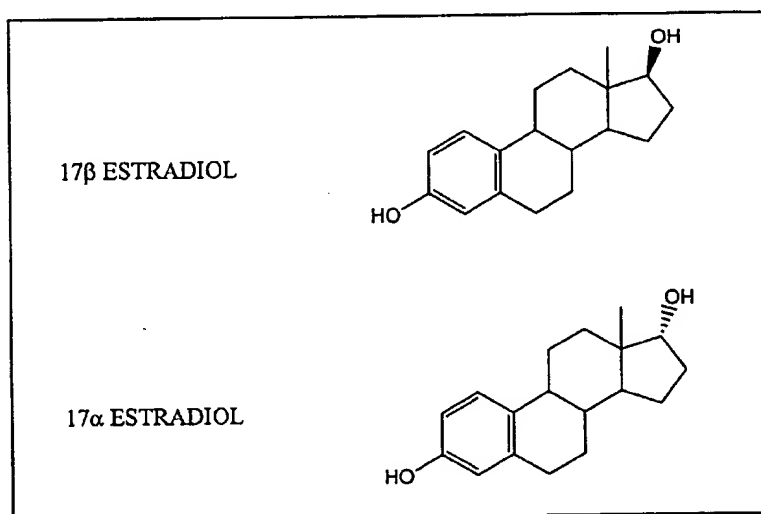


FIG. 17

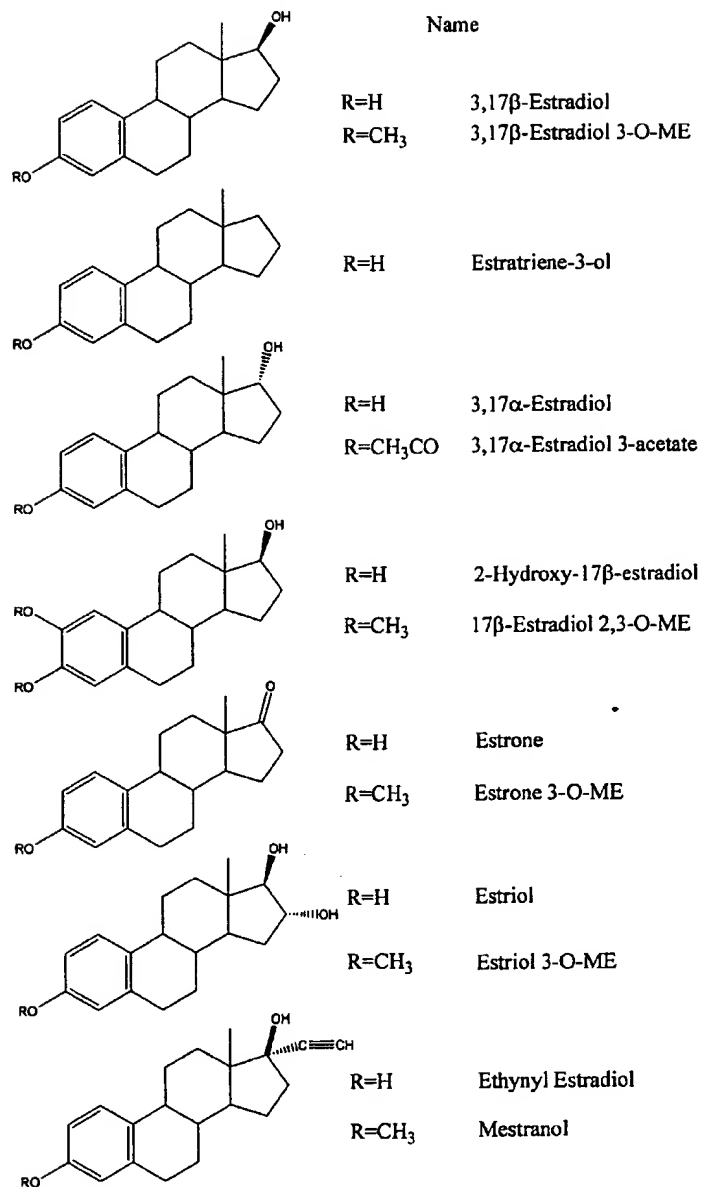


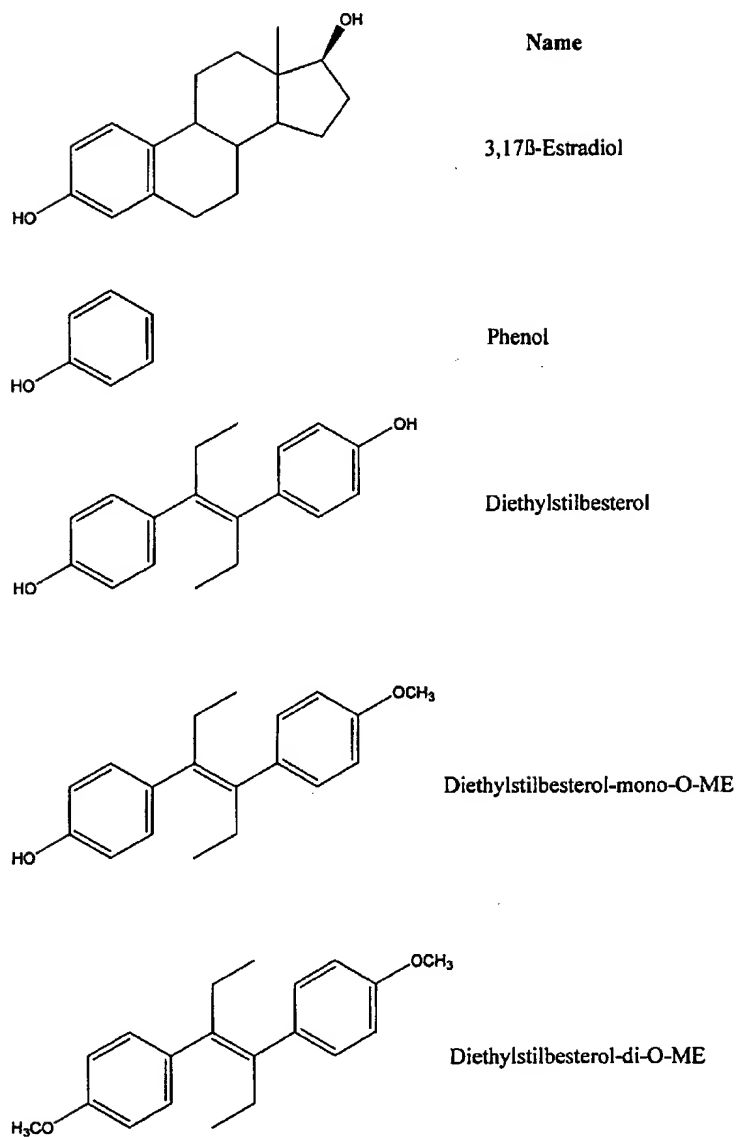
FIG. 18

FIG. 19

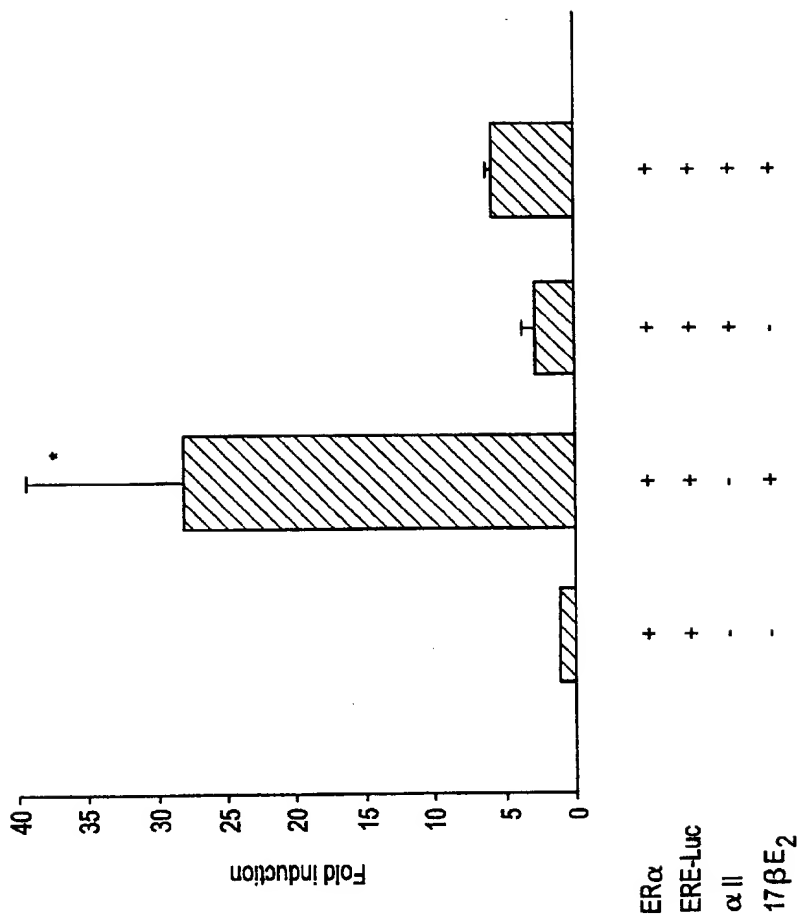


FIG. 20

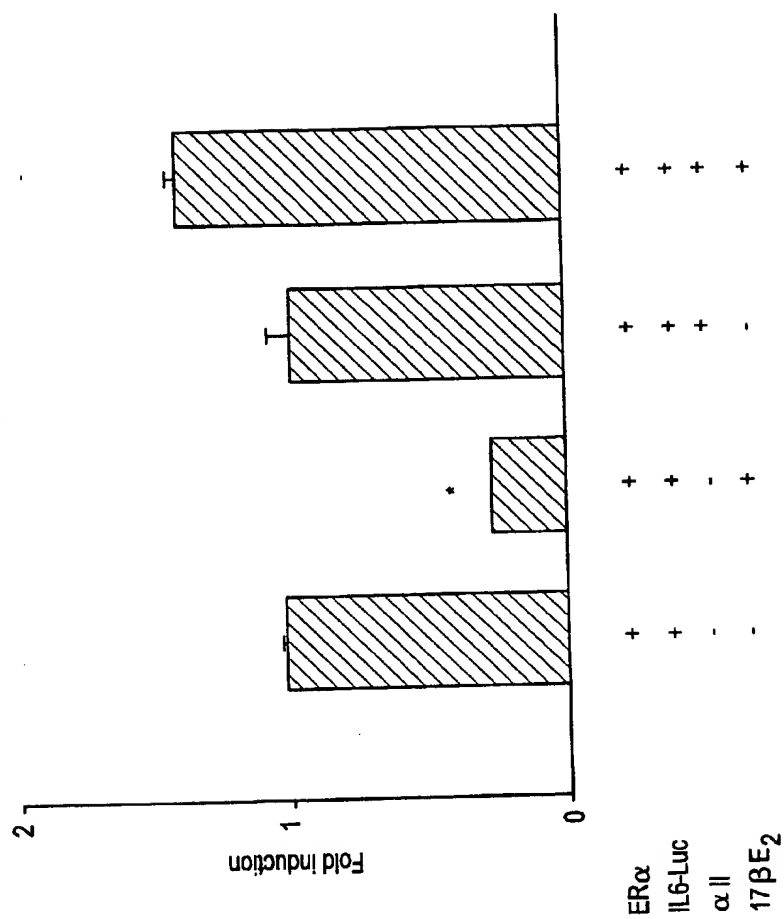
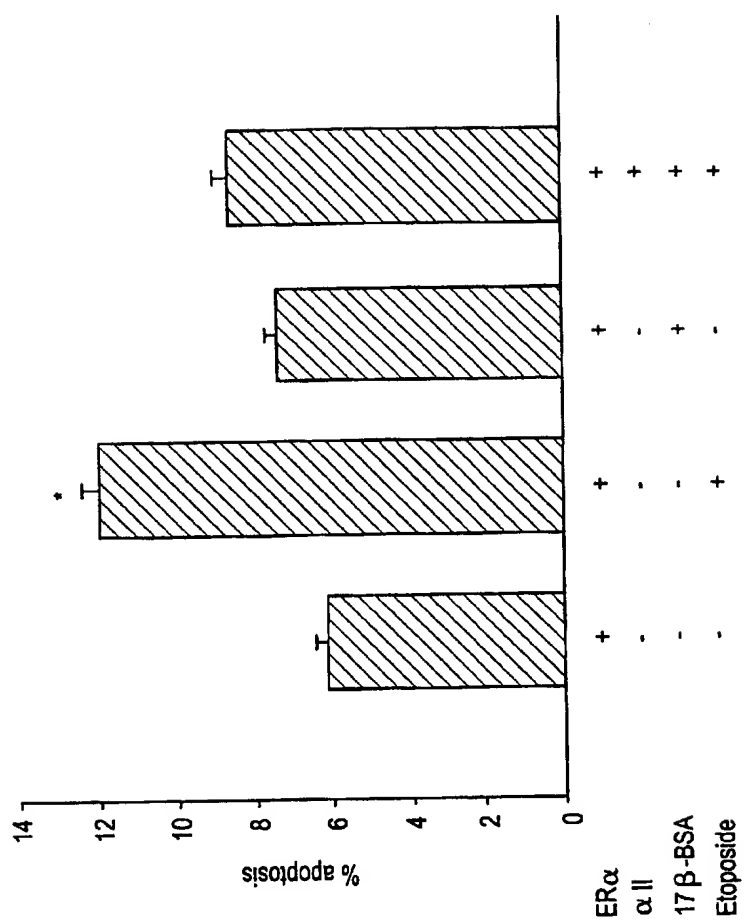


FIG. 21



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23355

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/56

US CL :514/179, 182

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/179, 182

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,189,212 A (RUENITZ) 23 February 1993, see particularly col. 1, lines 26-27 and claim 15.	1-45
X	US 5,362,720 A (LABRIE) 08 November 1994, see columns 7-9, particularly column 9, lines 44 and 45.	1-45

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

B earlier document published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

A document member of the same patent family

Date of the actual completion of the international search

16 DECEMBER 1999

Date of mailing of the international search report

04 FEB 2000

Name and mailing address of the ISA/US
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23355

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

BRS(WEST)

search terms: estradiol, estratriene, testosterone, dihydrotestosterone, dehydroisoandrosterone, androstenedione, androstenediol, RU 1881, osteoporosis, bone mass, Paget's Disease, etc.